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Temperature Dependence of Cholinesterase and Lactate Dehydrogenase in the Guinea-pig, Hedgehog and Codfish

By

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Abstract

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The temperature dependence of lactate dehydrogenase (LDH) in brain, heart and skeletal muscle and of acetylcholinesterase (AChE) in brain tissue was investigated in a homeothermic animal (the guinea pig), a poikilothermic animal (the codfish) and a hibernator (the hedgehog). The temperature of maximal LDH activity was higher in the tissues from the guinea pig than the other animals (Table I). Similar and more pronounced results were obtained for AChE (Table IV).

The coordinated physiological activity of a homeothermic animal is restricted to a comparatively small temperature range around 37° C although an organ such as the heart can function at a lower temperature. The heart of a guinea-pig for example, isolated and perfused with the Langendorff technique, keeps beating down to a temperature of 15—20° C. The heart of a poikilothermic animal such as the flounder fish beats at a lower temperature but the upper temperature limit is not as high as for a homeothermic heart. A hibernator such as the hedgehog shows a wider temperature range from just above zero to 35°—40° C (Biörck and Johansson 1955). The reasons for these differences in temperature resistance are not shown but differences in the temperature characteristics of various enzymes have been suggested as a factor of importance (Huttunen and Johansson 1963). To investigate this the temperature dependence curves of lactate dehydrogenase[1] and acetylcholinesterase[2] were studied in vertebrates with different temperature regulation: a homeothermic animal, a hibernator and a poikilothermic animal.

Material and methods

Guinea pigs were used to represent the homeothermic animals, hedgehogs (*Erinaceus europaeus*), non hibernating and hibernating, the hibernators and codfish the poikilothermic animals. 10, 9, 11 and 10 animals respectively were used for determinations of the lactate de-

[1] E.C. 1.1.1.28 D-lactate: NAD oxidoreductase
[2] E.C. 3.1.1.7 Acetylcholine hydrolase

hydrogenase (LDH) temperature dependency The corresponding numbers for acetylcholin esterase (AChE) were 10, 2 10 and 10 animals Heart, brain and skeletal muscle were used for the LDH studies but brain only for the AChE studies

The guinea pigs and the non hibernating hedgehogs were anesthetized with Nembutal (60 and 45 mg per kg b w respectively) The hibernating hedgehogs and the codfish were not anesthetized before the removal of the heart, brain and skeletal muscle These tissues were rinsed in saline and then kept at 0° C for 30 min until they were homogenized at room temperature for ten min and in a 0 9 % KCl solution using a homogenizer of the Potter Elvehjem type The amount of wet tissue per ml 0 9 % KCl were for AChE 200 mg brain tissue in all species while the corresponding amount of wet tissue in the LDH analyses were for heart tissue hedgehog 2 5 mg guinea pig 2 5 mg codfish 0 625 mg for skeletal muscle tissue hedgehog 5 mg, guinea pig 2 5 mg codfish 2 5 mg and for brain tissue 10 mg in all 3 species The homogenate was stored in a refrigerator at +4° C and used within 2 hrs The LDH activity was determined as described by Chefurka (1954) at 5°, 10° 15°, 20°, 25°, 30°, 35°, 40°, 45°, 50° 55° and 60° C in a Beckman DB spectrophotometer The activity at a certain temperature was determined as the difference in metabolized substrate in µmoles (values expressed on wet tissue weight) between the 6 and 0 min values after the addition of the pyruvate solution In homogenates from 5 non hibernating hedgehogs pH was changed with a phosphate buffer according to Sorensen from 5 2 to 8 8 and the activity was measured at 10°, 20° and 37° C AChE activity was observed only in the brain it was determined according to the method described by Meyer and Wilbrandt (1954) A slight modification was introduced acetylthiocholine iodide being used instead of butyrylthiocholine iodide Results were recorded as the difference in metabolized substrate in µmoles (values expressed on wet tissue weight) obtained *10* and *5* min as well as *30* and *25* min after the addition of acetylthiocholine iodide

The t test was used for the calculations of statistical significance

Results

Lactate dehydrogenase (LDH) The temperature of maximal LDH activity for the brain, heart and skeletal muscle was determined in each species The results are sented in Table I In the case of the hearts the temperature of maximal LDH vity was higher for the guinea pig than for the hibernating hedgehog and the dfish It was also significantly higher for maximal brain LDH activity in the uinea pig than in the hibernating hedgehog while the skeletal muscle from the hibernating hedgehog showed higher values than both the guinea pig and the cod fish

The difference between the maximal LDH activity and the activity obtained at 5° C was calculated as an estimation of the shape of the curve a high value indicating a peaked curve and a low value a shallow curve The result is given in Table II The codfish tissues showed low values indicating a small difference between the maximum activity and *the activity obtained at 5° C The heart and skeletal muscle* from guinea pig and hibernating hedgehog showed higher values while the guinea pig brain curve was rather shallow The lower values obtained for the non hibernating hedgehogs compared with the hibernating animals was an unexpected finding The activities of the hedgehog heart and skeletal muscle were largely unaffected by changes in pH from 5 2 to 7 6 while a fall in activity was observed at pH 8 0 and higher (Table III) The changes were more pronounced at 37° than at 10° C The hedgehog brain tissue showed a similar pattern but the fall in activity started at pH 7 6 Fewer observations were made on guinea pig tissue but the results seemed consistent with those found in the hedgehogs

TABLE I Temperature in degrees centigrade for maximal lactate dehydrogenase (LDH) activity in the heart, skeletal muscle and brain from guinea pig non hibernating and hibernating hedgehog and codfish

M, mean *n*, number of observations *SD*, standard deviation The P values for the differences between the various species are also given See further the text

	Guinea pig			Non hibernating hedgehog			Hibernating hedgehog			Codfish		
	M	n	SD	M	n	SD	M	n	SD	M	n	SD
Heart	45.0	10	2.4	44.0	5	4.2	42.7	11	2.6	40.5	10	5.0
Skeletal muscle	35.0	10	4.1	33.0	5	5.7	39.5	11	4.2	34.5	10	5.0
Brain	42.0	10	3.5	32.0	5	4.5	34.0	10	4.2	38.0	10	7.5

Difference

	Guinea pig — hibernating hedgehog	Guinea pig — codfish	Hibernating hedgehog — codfish
Heart	0.05 P > 0.01	0.05 > P > 0.01	P 0.05
Skeletal muscle	0.05 P 0.01	P > 0.05	0.05 > P 0.01
Brain	P 0.001	P > 0.05	P 0.05

TABLE II Difference between maximal value and value obtained at 5 C for lactate dehydrogenase LDH activity in the heart skeletal muscle and brain from guinea pig non hibernating hedgehog and codfish See further table I for legend

	Guinea pig			Non hibernating hedgehog			Hibernating hedgehog			Codfish		
	M	n	SD	M	n	SD	M	n	SD	M	n	SD
Heart	0.055	8	0.009	0.052	4	0.007	0.0 0	10	0.00	0.045	10	0.009
Skeletal muscle	0.055	8	0.007	0.038	4	0.008	0.0 8	10	0.016	0.035	10	0.009
Brain	0.035	8	0.018	0.037	4	0.005	0.041	10	0.018	0.040	10	0.010

Difference

	Guinea pig hibernating hedgehog	Guinea pig codfish	Hibernating hedgehog codfish
Heart	0.01 P 0.001	0.05 P 0.01	P 0.001
Skeletal muscle	P 0.0	P 0.001	0.01 P > 0.001
Brain	0.01 P 0.001	P 0.05	0.01 P 0.001

TABLE III Lactate dehydrogenase (LDH) activity in the heart, skeletal muscle and brain at 10° C,

	pH	5.2			5.6		
		M	n	SD	M	n	SD
	10° C	0.0218	4	0.0119	0.0236	4	0.0125
Heart	20° C	0.0260	5	0.0113	0.0269	5	0.0127
	37° C	0.0376	5	0.0129	0.0371	5	0.0086
	10° C	0.0271	4	0.0135	0.0294	4	0.0148
Skeletal muscle	20° C	0.0313	5	0.0149	0.0310	5	0.0151
	37° C	0.0393	5	0.0109	0.0392	5	0.0077
	10° C	0.0171	4	0.0085	0.0184	4	0.0086
Brain	20° C	0.0258	5	0.0133	0.0260	5	0.0137
	37° C	0.0359	4	0.0148	0.0371	4	0.0133
	pH	**7.2**			**7.6**		
		M	n	SD	M	n	SD
	10° C	0.0237	4	0.0104	0.0236	4	0.0095
Heart	20° C	0.0303	5	0.0063	0.0303	5	0.0079
	37° C	0.0408	5	0.0144	0.0338	5	0.0159
	10° C	0.0276	4	0.0108	0.0241	4	0.0089
Skeletal muscle	20° C	0.0367	5	0.0140	0.0318	5	0.0167
	37° C	0.0376	5	0.0133	0.0260	5	0.0108
	10° C	0.0169	4	0.0063	0.0138	4	0.0038
Brain	20° C	0.0286	4	0.0094	0.0222	5	0.0066
	37° C	0.0325	4	0.0186	0.0205	4	0.0084

Acetylcholine esterase (AChE) Maximal AChE activity was obtained at a higher temperature in brain tissue from guinea-pigs than in corresponding tissue from cod fish, while the hibernating hedgehogs showed intermediate values (Table IV) This was so both for the 10—5 and for the 30—25 min values

The shape of the curve was calculated in the same way as for LDH as the difference between the maximal AChE activity and the activity obtained at 5° C No difference was found between guinea pigs and hibernating hedgehogs (Table V) while the codfish difference was smaller

Discussion

One reason for the varying temperature sensitivity in homeothermic and poikilothermic animals may be differences in physico-chemical properties represented for example by the different melting points of fats This is supported by the studies of

20° C and 37° C from a non hibernating hedgehog. See further table I for legend and the text

6.0			6.4			6.8		
M	n	SD	M	n	SD	M	n	SD
0.0233	4	0.0127	0.0230	4	0.0112	0.0224	4	0.0099
0.0265	5	0.0117	0.0261	5	0.0107	0.0313	5	0.0169
0.0399	5	0.0154	0.0400	5	0.0141	0.0465	5	0.0111
0.0280	4	0.0145	0.0286	4	0.0133	0.0294	4	0.0131
0.0337	5	0.0166	0.0347	5	0.0162	0.0371	5	0.0161
0.0421	5	0.0139	0.0460	5	0.0139	0.0467	5	0.0102
0.0171	4	0.0083	0.0181	4	0.0082	0.0182	4	0.0069
0.0277	5	0.0144	0.0296	5	0.0149	0.0307	5	0.0145
0.0395	4	0.0195	0.0424	4	0.0213	0.0407	4	0.0155

8.0			8.4			8.8		
M	n	SD	M	n	SD	M	n	SD
0.0220	4	0.0069	0.0171	4	0.0009	0.0143	4	0.0027
0.0267	5	0.0108	0.0193	5	0.0067	0.0148	4	0.0064
0.0225	5	0.0154	0.0111	5	0.0042	0.0051	4	0.0041
0.0243	4	0.0075	0.0193	4	0.0043	0.0116	4	0.0062
0.0315	5	0.0159	0.0225	5	0.0094	0.0127	4	0.0081
0.0148	5	0.0064	0.0076	5	0.0014	0.0029	4	0.0022
0.0117	4	0.0035	0.0061	4	0.0024	0.0039	4	0.0023
0.0188	5	0.0086	0.0087	5	0.0022	0.0057	4	0.0025
0.0081	4	0.0093	0.0030	4	0.0034	0.0003	3	0.0003

Fawcett and Lyman (1954) and *Huttunen and Johansson* (1963). Another factor might be differences in the properties of enzymes. This is supported by the findings in the present paper. Other factors are probably also involved. There were differences found in the temperature for maximal AChE activity, higher values being obtained in brain from guinea-pig than from codfish and intermediate values in the hibernating hedgehog. This fits in well with the data presented in the introduction.

The changes in LDH activity were not as consistent as for AChE. It is reasonable that this temperature dependence would be more pronounced in the evolutionarily "young" enzymes. LDH is presumably a comparatively "old" enzyme from an evolutionary point of view.

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The valuable technical assistance of Miss Barbro Eklund is gratefully acknowledged.

TABLE IV Temperature in degrees centigrade for maximal acetylcholine esterase (AChE) activity in the brain from guinea pig, hibernating hedgehog and codfish The table gives the differences between the activity after 10 and 5 minutes and after 30 and 25 minutes

M, mean, n number of observations, SD standard deviation The P values for the differences between the various species are also given

	Guinea pig			Hibernating hedgehog			Codfish		
	M	n	SD	M	n	SD	M	n	SD
10—5 minutes	46 0	10	4 6	39 5	10	7 1	31 8	10	4 7
30—25 minutes	45 8	*10*	*9 0*	*41 3*	*10*	*5 4*	*33 8*	*10*	*3 6*

Difference

	Guinea pig — hibernating hedgehog	Guinea pig — codfish	Hibernating hedgehog — codfish
10—5 minutes	0 05> P> 0 01	P <0 001	0 05> P> 0 01
30—25 minutes	*P> 0 05*	*0 01> P> 0 001*	*0 01 > P> 0 001*

TABLE V Difference between maximal activity and activity at 5 C for acetylcholine esterase (AChE) in brain tissue obtained from guinea pig hibernating hedgehog and cod fish

See further table IV for legend and the text

	Guinea pig			Hibernating hedgehog			Godfish		
	M	n	SD	M	n	SD	M	n	SD
—5 minutes	0 94	10	0 22	0 95	10	0 23	0 68	10	0 11
—25 minutes	0 69	10	0 25	1 10	10	0 62	0 44	10	0 19

Difference

	Guinea pig — hibernating hedgehog	Guinea pig — codfish	Hibernating hedgehog—codfish
10—5 minutes	P> 0 05	0 01 > P 0 001	0 01> P> 0 001
30—25 minutes	P> 0 05	0 05 P 0 01	0 01> P 0 001

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Pulmonary Function and G-Stress during Inhalation of 100 % Oxygen

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Abstract

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The effect of 3 min exposure to 5 G acting in the head to seat direction on arterial oxygen saturation (S_{aO_2}) and pH inspiratory minute volume (V_I) end tidal P_{CO_2} and heart rate was studied in 8 healthy subjects breathing 100 % oxygen and wearing anti G suits During the first min S_{aO_2} remained unchanged Thereafter it fell at varying rates in different subjects the average reduction at the end of the exposure being 6 % (range 0—17 %) Mean V_I showed a rapid initial increase by 3 liters min remained thereafter essentially unchanged for approximately one min and increased continuously during the last two min (average final increase 7 liters/min) There were considerable indi

It is well known, that increased gravitational stress may produce gross shunting of blood in dependent parts of the lungs, leading to insufficient oxygenation of the arterialized blood (Barr, Bjurstedt and Coleridge 1959 Barr 1962 Wood *et al* 1963) In a previous investigation from this laboratory, Barr (1963) studied the effects of exposing human subjects breathing air to a force of 5 G caused by headward acceleration (effective force acting in the head to seat direction) A 4 fold increase of the alveolar arterial O_2 difference was found and in spite of concomitant hyperventilation, an average reduction occurred in the arterial oxygen saturation by 8 8 per cent These alterations started almost immediately after the onset of acceleration, 90 per cent of the final changes being attained within less than one min

The effects of oxygen inhalation on the arterial oxygen saturation during exposures to transverse accelerations effective G force acting transversely to the long axis of the body) have been studied by serveral investigators Wood *et al* 1963, Hoppin Sever and Hitchcock 1965, Banchero *et al* 1967 However, corresponding st

during headward acceleration, with the circulatory system exposed to much larger hydrostatic pressure differences, are largely lacking

The present investigation was therefore undertaken to study, in human subjects, the effect of the breathing of 100 % oxygen at normal atmospheric pressure on the pulmonary gas exchange during headward acceleration For this purpose, the subjects were exposed to a force of 5 G acting in the head to seat direction while pulmonary ventilation, end tidal P_{CO_2} and arterial oxygen saturation and pH were continuously and simultaneously recorded

Methods

Eight healthy fighter aircraft navigators served as test subjects *All were familiar with the procedures* used and with the subjective sensation experienced in centrifuge experiments Age and other individual data are given in Table I

All subjects were studied in the seated position in the cabin of a human centrifuge (for description of the centrifuge, see Gotzlinger and Helsing 1955, Bjurstedt 1957) They were supported by a backrest inclined 13 from the vertical On starting the centrifuge, the cabin swings out freely so that, when constant speed has been attained the resultant force has the same direction in relation to the body axis as the normal force of gravity with the centrifuge standing still In the present *experiments the subjects were exposed* at unchanged body position, to a force of five times that exerted by normal gravity (headward acceleration of 5 G, often denoted as +5 G_z) The duration of the exposure was 3 min which included some 12—15 sec used for the increase of the magnitude of the G force from 1 G (centrifuge stationary to 5 G

During and for at least 10 min before the exposure, virtually *100 %* oxygen was supplied to the airways via a modified tight fitting aviator's oxygen mask, and a demand regulator The subjects used automatically inflated anti-G suits

Throughout the experiments arterial pH and oxygen saturation total ventilation end tidal P_{CO_2} and heart rate were recorded continuously A Teflon catheter was introduced percutaneously into the subject's left radial artery at the wrist Barr 1961 and blood was drawn continuously through the sensing units at a constant rate of 8 0 ml min by means of a roller pump 150 mg Heparin Vitrum was given intravenously to prevent clotting of the blood.

Details of recordings and laboratory methods have been described in a previous report Barr 19[illegible]3)

Expired minute volume was recorded from a dry rotary gas meter inserted between the demand regulator and a respiratory valve connected to the aviator's mask (external dead space = 30 ml)

End-tidal P_{CO_2} was automatically obtained by employing a breath by breath" gas-sampling device [illegible]mar Hesser and Matell 1962 and a CO_2 infrared analyzer

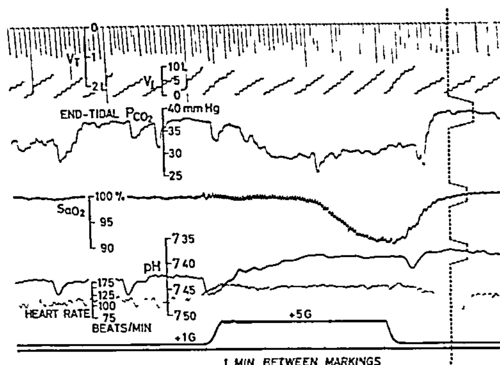
Arterial oxygen saturation and pH were recorded by a cuvette oximeter Wood Geraci and Groom 1948 and a glass reference electrode assembly previously developed by Barr and Bjurstedt (see Barr et al 1964

Heart rate was recorded by an instantaneous cardiotachometer Sturm and Wood 19[illegible]

All variables studied were continuously recorded on photokymographic paper at a speed of 1.27 mm sec via light beam galvanometers

TABLE I Physical characteristics of subjects

Subject	Age years	Height cm	Weight kg	Blood pressure mm Hg)	Hb conc g 100 ml blood
AS	34	173	82	135 90	15 1
AO	38	180	78	140 95	15 4
AJ	24	190	83	125 85	14.8
PL	33	191	92	140 90	15 7
LH	27	181	79	140 80	14 5
NH	32	173	73	140 95	15 0
HG	24	170	60	145 90	15 1
ML	29	180	70	130 90	13 9



Results

Fig 1 exemplifies the type of photokymographic recordings from which the primary data were obtained Individual time averages determined over 1 and 2 min periods before, during and after the exposure to 5 G are shown in Table II The changes observed have been summarized in Fig 2, where the group means of individual time averages over successive 15-sec intervals have been plotted against time The values for ventilation were smoothed by determining, for every 15 sec, the mean of three consecutive values For comparison, the corresponding results from earlier experiments in which the subjects breathed air (Barr 1963) were plotted in the same figure Fig 3 presents the time-courses of the individual, G-induced deviations from the resting values

The exposure to 5 G was tolerated by all subjects without blackout or other symptoms of arterial hypotension at head level All subjects reported, however, a progressively increasing feeling of fatigue During the first few min after the exposure, 3 of the 8 subjects experienced substernal pain during deep inspirations, and fits of coughing occurred occasionally in four subjects

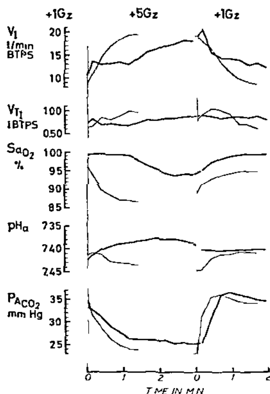


Fig 2

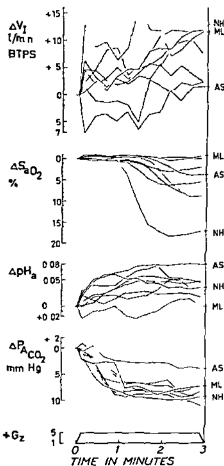


Fig 3

ages determined over the last 15 sec period before the acceleration exposure *Note* the opposite changes in the arterial pH during oxygen breathing and air breathing and the markedly smaller ventilatory increase during the first min at 5 G when breathing oxygen

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Pulmonary ventilation (V_I) Following the ean value for V_I showed a rapid initial increase and anged for approximately one min, about 3 2) Taken on the basis of individual ure (Fig 3) During the first min *AS* while subject *ML* at the sam tion of the t test to the intraind

TABLE II Effects of 3 min exposures to 5 G during inhalation of 100 % oxygen Individual averages over specified periods of sampling (Heart rate in beats/min)

Subject		2—0 min before exposure	0—1 min during exposure	2—3 min during exposure	1.5—2 5 min after end of exposure
AS	O_2 sat, %	99 5	99 9	96 3	99 8
	pH	7 465	7 416	7 385	7 397
	V_I, l/min	14 14	9 24	14 86	12 68
	heart rate	73	113	123	72
AO	O_2 sat, %	99 6	100 1	93 3	99 8
	pH	7 419	7 403	7 367	7 385
	V_I, l/min	9 20	12 63	17 47	13 32
	heart rate	71	100	125	77
AJ	O_2 sat, %	99 8	99 6	97.2	99 9
	pH	7 407	7 370	7 356	7 381
	V_I, l/min	10 02	13 93	19 72	12 49
	heart rate	74	114	124	73
PL	O_2 sat, %	99 3	99 1	91 4	98 3
	pH	7 437	7 416	7 385	7 397
	V_I, l/min	11 87	11 35	13 77	8 51
	heart rate	108	145	144	110
LH	O_2 sat, %	99 4	99 2	93 6	99 9
	pH	7 426	7 413	7 403	7 390
	V_I, l/min	10 79	16 82	22 90	15 47
	heart rate	71	104	119	73
AH	O_2 sat, %	99 6	99 8	81 9	97 9
	pH	7 401	7 355	7 365	7 362
	V_I, l/min	*11 74*	*13 58*	*22 60*	*10 59*
	heart rate	77	98	110	83
HG	O_2 sat, %	99 4	100 0	99 4	100 0
	pH	7 430	7 415	7 385	7 418
	V_I, l/min	9 17	12 65	13 03	13 51
	heart rate	78	125	148	103
ML	O_2 sat, %	99 1	99 2	99 5	99 6
	pH	7 398	7 408	7 390	7 416
	V_I, l/min	6 81	16 23	16 29	11 27
	heart rate	76	103	106	82
Means	O_2 sat, %	99 5	99 6	94 1	99 4
	pH	7 423	7 400	7 380	7 393
	V_I, l/min	10 5	13 3	17 6	12 2
	heart rate	78 5	112 8	124 9	84 1
SD	O_2 sat, %	0 2	0 4	5 7	0 8
	pH	0 022	0 024	0 016	0 018
	V_I, l/min	2 21	2 47	3 82	2 10
	heart rate	12 2	15 8	14 7	14 5

control value (*cf* Table II) revealed however a statistically significant increase by 2 8 liters/min ($P<0\ 01$) As shown in Fig 2 the average increase in $\dot{V}_I$ during the first min was considerably smaller than was the case in earlier experiments when air was breathed During the last two min of the acceleration exposure $\dot{V}_I$ increased progressively in all subjects, and the average 3rd min value was significantly higher than the 1st min value ($P<0\ 001$)

The arterial oxygen saturation (S_{aO_2}) remained essentially unchanged in all subjects during the first min of acceleration (Fig 3) Thereafter, S_{aO_2} fell at varying rate in different subjects, an apparent stabilization being attained during the third min In two of the cases, however, S_{aO_2} remained practically unchanged at the 99—100 per cent level during the entire 3 min exposure One subject, on the other hand, showed a maximal reduction by as much as 19 per cent

Fig 2 illustrates that at 5 G the breathing of oxygen as compared to air breathing both delayed the onset of arterial desaturation and reduced its severity These observations agree with earlier findings during transverse accelerations (Wood *et al* 1963, Hoppin, Sever and Hitchcock 1965)

Arterial pH The group mean value showed a gradual decrease by 0 04 pH units during the first 2 min at 5 G and then increased slightly during the last min (Fig 2) Both the 2nd and 3rd min values showed statistically significant reductions below the control value $P<0\ 001$ This response differs from that earlier obtained in subjects breathing air who showed a small but statistically significant shift towards alcalosis
e of the subjects in the present study *ML* differed from the others in showing a light increase in the pH Table II) The same subject also showed a markedly greater increase in the respiratory minute volume during the first 2 min of the G stress than did the rest of the subjects see Fig 3)

End tidal P_{CO} The observed changes were less marked but similar to those earlier obtained in subjects breathing air Fig 2 An average reduction of about 7 mm Hg was attained within approximately one min at 5 G and all subjects showed only *slight changes thereafter*

Subject *AS*, in whom the ventilation in contrast to the general pattern decreased (see above), showed the smallest reduction in end tidal P_{CO_2} and also the greatest fall in arterial pH see Fig 3 His control values shown in Table II indicate that this response pattern at least partly can be explained by hyperventilation prior to the acceleration exposure, the control values for both respiratory minute volume and pH being markedly higher Table II and his end tidal P_{CO_2} lower, than in the rest of the subjects

Discussion

The disturbances in pulmonary function which occur with the exposure to G forces in the head to seat direction for reviews see Wood *et al* 1963 b Bjurstedt 1964) are attributed mainly to the increased effective weight of the blood which is displaced

downwards in both the pulmonary and systemic circuits The resulting changes in the flow and volume distribution of blood in the lungs cause an "alveolar dead space" i e overventilation (high V/Q ratios) in the upper regions and a "venous admixture" i e overperfusion (low V/Q ratios) in the basal parts In previous experiments at +5 G_z on subjects breathing air and using anti G suits, large alveolar to arterial (A−a) differences for both P_{O_2} and P_{CO_2} were demonstrated as a consequence of these changes (Barr 1963), and a marked fall occurred in the arterial oxygen saturation

The present finding that an arterial oxygen desaturation during headward acceleration occurs also during the breathing of oxygen is in agreement with earlier observations in experiments on dogs (Barr, Bjurstedt and Coleridge 1959) A considerable A−a O_2 difference due to venous admixture is indicated by this desaturation

It might be expected that in lung areas with low V/Q ratios the inhalation of oxygen would result in a more complete saturation of the hemoglobin than when air is breathed Evidently in the present experiments this effect of oxygen did not cancel the G induced venous admixture effect on the arterial oxygen saturation A possible explanation may be that the venous admixture during both oxygen and air breathing is primarily caused by collapse of alveoli in dependent lung regions (V/Q ratios − zero), and that hypoventilation (V/Q ratios low but greater than zero) and impairment of diffusion plays only minor role This was suggested by Banchero *et al* (1967) to be the case in dogs undergoing transverse acceleration, in which breathing 99 6 per cent oxygen was found not to prevent arterial desaturation and in which the estimated total pulmonary arterial venous shunt was similar to that when the animals breathed air

In lung regions whose function can be improved by high oxygen tensions, the extent to which the increased alveolar P_{O_2} during oxygen breathing can rise the oxygen content of the blood leaving these areas is limited due to the flat top of the oxygen dissociation curve Therefore, the higher oxygen content of the blood in these areas must have been far from sufficient to compensate for the reduction in oxygen content which occurred along the steep part of the oxyhemoglobin curve in lung regions with V/Q ratios approaching zero

During air breathing the presence of nitrogen in alveoli behind an airway obstruction tends to prevent the collapse of these alveoli (*cf* Rahn and Farhi 1963) Therefore, atelectasis can be expected to develop more rapidly during oxygen breathing In fact it has been stated (Hyde, Pines and Saito 1962) that in conjunction with acceleration the breathing of 100 % oxygen is a prerequisite for the development of atelectasis It can not be excluded therefore, that the breathing of oxygen in the present study caused atelectasis to be more extensive than it would have been with air

The observed delay in the onset of the arterial desaturation can be explained if it is assumed that G induced alveolar collapse is not only immediate but occurs also secondary to distal air way closure, with gas trapping and delayed absorbtional atelectasis (*cf* Vandenberg *et al* 1968) Also, transudation of fluid into basal alveo and resulting diffusion impairment may have been progressive

Efficiency of Ventilation

The considerable individual differences in the ventilatory response shown in Fig 3 may well partly be explained by psycogenic factors, e g reflexes conditioned in previous aerobatic training However the V_I plot in Fig 2, which was based on the group means of individual time averages, presents changes that were statistically significant see Results , viz an initial, rapid increase and a second, more gradual increase after about one min

The increase in ventilation which occurs with the exposure to 5 G during both oxygen and air breathing can partly be explained by identical, G induced mechanisms, viz the combined influences of reduced cardiac output and arterial hypotension in the upper part of the body, directly on the medullary respiratory centers and through reflexes from the carotid chemoreceptors and baroreceptors (for review, see Rosenhamer 1967 The initial ventilatory increase was, however, much smaller during oxygen breathing than during air breathing (see Fig 2) This difference in the response pattern probably represents the role that arterial hypoxemia plays in the G induced chemoreceptor reflex stimulation of the respiration The significant increase in the respiratory minute volume which occurred after about 1 min during oxygen breathing can analogously be explained by the decrease in the arterial oxygen saturation which occurred by this time

The changes observed in the arterial pH in the present experiments were relatively ll, amounting on an average to only 0 04 of a pH unit in the acid direction This elative stability in pH tallies with previous findings at 5 G during air breathing Barr 1963 Under the last mentioned conditions a rather marked alveolar dead space was found This is attributed to 1 downward displacement of blood in the pulmonary circulation and 2 closing of pulmonary capillaries secondary to decreased cardiac output Since there are no obvious reasons to assume that the inhalation of 100 per cent oxygen would appreciably affect these events, a significant alveolar dead space probably occurred in the present experiments as well In the absence of a concomitant increase in total ventilation such dead space would tend to decrease the arterial pH Therefore, the observed moderate acidotic shift in the present study indicated that the inital ventilatory increase which occurred in the absence of the respiratory drive of arterial hypoxemia was insufficient to compensate for the G induced dead space effect In fact, the greater increase in ventilation which accompanies the exposure to 5 G when air is breathed Barr 1963, see Fig 2), results in a small alkalotic shift As can be seen in Fig 2 (heavy line , the moderate acidotic shift in the present experiments levelled off following the increase in ventilation which occurred after about one min at 5 G, and there was even a slight alkalotic trend at the end of the exposure by which time the ventilation had reached almost the same value as that attained during air breathing in the previous experiments

A contributing factor to the occurrence of the small acidotic shift during oxygen breathing in contrast to air breathing may have been the impairment of the CO_2 removal from the blood that results from a greater contribution to the total venous shunt from atelectatic alveoli (V/Q ratios=zero, see the above discussion)

G induced redistribution of blood and respiratory responses

The individual changes in subject *NH* and *ML* seem to represent two different types of adaptation of the pulmonary function to exaggerated G stress As shown in Fig 3, subject *NH* showed an only small ventilatory increase during the first min of the G exposure It can also be seen in Fig 3 that during this time there was a comparatively marked fall in the arterial pH, indicating that the ventilatory increase was too small to compensate for the G induced alveolar dead space That on the other hand, the venous admixture effect must have been unusually large in this subjects was indicated by the fact that he showed a much greater reduction in the arterial oxygen saturation than did the rest of the subjects (Fig 3)

In contrast to *NH*, subject *ML* showed a very marked ventilatory increase during the first min of acceleration Unlike the rest of the subjects he also showed a slight increase in the arterial pH (Fig 3) Furthermore, as shown in Fig 3, arterial oxygen desaturation did not occur in subject *ML*, indicating a relatively smaller venous admixture effect than in the majority of the subjects

All subjects were well acquainted with the experimental procedure, and prior to G-exposure there were no signs of apprehension or anxiety such as hyperventilation or increase in heart rate in either *NH* or *ML* hence the much smaller initial ventilatory increase in *NH* as compared to *ML* may be attributed to a less pronounced respiratory stimulation by the G factor itself, although psychogenic factors may also have played a role The underlying mechanism may well have been a less marked displacement of blood volume from the upper to the lower body regions in *NH*, possibly due to a greater effectiveness of the G-suit, leading to a better maintenance of the blood flow at the level of the head including the respiratory centers and the carotid sinus region Thus, the observation of an only small ventilatory increase initially would be consistent with the retention of a relatively large volume of blood in the upper part of the body, including the pulmonary circulation It can be inferred furthermore, that this large blood volume in the dependent lung regions during increased gravitational stress tends to enhance venous admixture effects due to atelectasis (Rosenhamer 1969) This is analogous to the increased venous admixture during G stress previously demonstrated to result both from the use of an anti G suit (Barr 1963 a), which limits the drain of blood away from the upper body parts, and from en exercise induced redistribution of blood into the thoracic spaces (Rosenhamer 1967) Therefore, both the greater initial ventilatory increase and the absence of a final arterial oxygen desaturation in *ML* in contrast to *NH* can be attributed to a more marked G induced displacement of blood volume from the upper to the lower body parts

The lower pH in *NH* as compared to *ML*, especially during the first half of the exposure is compatible with the assumption of more extensive complete atelectasis in dependent lung regions of *NH*, leading to an impaired CO_2 elimination from the blood More likely, however, the higher pH in *ML* resulted from the greater initial ventilatory increase, as the accompanying changes in end tidal P_{CO_2} Fig 2) do not indicate a significantly smaller A—a CO_2 difference in *ML* than in *NH*

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Acta physiol scand 1969 77. 17—22

From the Department of Physiology, University of Göteborg Sweden

Effect of Reflexes of Somatic Afferents on the Adrenergic Outflow to the Stomach in the Cat

By

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Received 18 November 1968

Abstract

JANSSON G *Effect of reflexes of somatic afferents on the adrenergic outflow to the stomach in the cat* Acta physiol scand 1969 77 17—22

The adrenergic activity to the stomach in cats anesthetized with chloralose was modified by electric stimulation of somatic afferent nerves. Electric stimulation of "somatic pressor" afferents promptly elicited inhibitory responses of stomach contractions which contractions were [illegible]

—Activation of "somatic depressor" afferents on the other hand suppressed the prevailing supply if any of adrenergic nervous activity to the stomach which could result in augmentation of vagally induced gastric motor responses Similar effects were obtained also after spinal cord transection indicating that the "somatic depressor" afferents made propriospinal reflex connections with the sympathetic outflow to the stomach

The adrenergic control of stomach motility seems to operate mainly or only as an antagonistic influence on the cholinergic activity in the stomach Thus clear demonstration of adrenergic inhibitory effects on stomach motility requires existing cholinergic nervous activity Such cholinergic nervous tone can be markedly and promptly inhibited by stimulation of the adrenergic supply to the stomach either reflexly from abdominal structures (Jansson and Martinson 1966) or from hypothalamic sympatho-excitatory structures (Jansson Lisander and Martinson 1969)

On the other hand electric stimulation of a hypothalamic sympatho-inhibitory structure can markedly augment cholinergically induced stomach contractions by suppressing a prevailing sympathetic inhibitory influence (Jansson Lisander and Martinson 1969)

Various groups of afferent somatic nerve fibres can reflexly exert a strong influence on the autonomic control of the circulation (Johansson 1962 Johansson Lundgren and Mellander 1964) Group III afferents from muscle can produce pronounced depressor responses by eliciting a reflex inhibition of sympathetic tone ('somatic depressor' fibres) while stimulation of afferent C fibres increases sympathetic discharge to the cardiovascular system ('somatic pressor' fibres)

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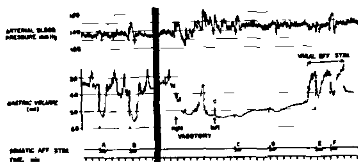


Fig 1 Cat 3 1 kg Inhibition of gastric motility on somatic "pressor afferent stimulation [illegible]
nerve stimulation A, D E, hamstring nerve stimulation B, C, F, 30 imp/sec, 3 msec, 15 V

tric stimulation of the vagal ' high-threshold relaxatory ' fibres to the stomach (*cf* Jansson and Martinson 1965)

After vagotomy the reflex gastric inhibition on "somatic pressor" stimulation again became pronounced provided a continuous vagal excitatory fibre stimulation had been started to re-establish the cholinergic excitation of stomach motility (Fig 1 E, F) The reflex inhibitions, induced by "somatic pressor' afferents and apparently mediated *via* the splanchnic nerves to the stomach thus appeared to exert their action by suppressing more or less selectively a cholinergic influence on stomach motility rather than by any direct effect on the myogenic tone of the stomach

2 *Effect of stimulation of "somatic depressor" afferents* In accordance with the findings by Johansson (1962), depressor responses were obtained by stimulation of somatic afferents at low frequencies (2—20 imp/sec) and at a voltage and pulse duration of about 2—8 V and 0 1—1 msec, respectively When the vagal nerves had been transected, stimulation of "somatic depressor' fibres had little or no effect on stomach motility

However, when the excitatory cholinergic fibres were stimulated in efferent direction and this excitatory vagal effect was counteracted by reflex activation of the adrenergic supply to the stomach (intestinal distension or afferent stimulation mesenteric nerve fibres), "somatic depressor' fibre stimulation result creased motor activity of the stomach This was also the case during

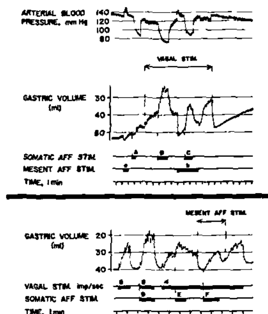


Fig 2 Cat 2 7 kg Effects of somatic "depressor" afferent stimulation on stomach motility in a vagotomized and adrenalectomized cat

Upper panel Somatic afferent stimulation elicited augmentation of vagally induced stomach contractions (A) also during mesenteric afferent stimulation (C) (Vagal stimulation 3 imp/sec, 2 msec, 5 V)

Lower panel Recording of stomach motility about 3 hours after spinal cord transection between C6 and C7 Note the augmentations of vagally induced excitatory motility Hamstring nerve stimulation 10 imp/sec, 1 msec, 8 V, mesenteric afferent stimulation 10 imp/sec, 3 msec, 4 V

after abdominal surgery as well as in comparatively lightly anesthetized animals, when the reflex activation of the adrenergic discharge to the stomach is fairly intense ...son and Lisander 1969) Fig 2 illustrates such an experiment in which efferent ...al fibres were stimulated in an animal where both vagal nerves had been cut ...s stimulation produced a moderate excitatory stomach motor response which was considerably enhanced by somatic depressor fibre stimulation Somatic depressor stimulation alone had no such effect When the flow of "background' reflex adrenergic activity to the stomach was blocked by guanethidine, the same vagal stimulation reduced the gastric volume much more and superimposed somatic depressor fibre stimulation then had no effect not even when submaximal vagal frequencies were used

The augmentatory motor response of the stomach induced by somatic depressor activation thus seemed to be due to suppression of an inhibitory adrenergic discharge, a suppression which, however was demonstrable only when the adrenergic fibres were allowed to suppress a prevailing cholinergic influence This thus showed that all reflex effects of somatic depressor fibres on stomach motility after vagotomy are abolished if the adrenergic effect is eliminated, *e g* by guanethidine Atropine also blocked the responses to stimulation by somatic depressor fibres since the presence of excitatory cholinergic nervous tone is necessary for the reflex adrenergic inhibitory effect to exert its influence The effect of stimulation of somatic depressor fibres persisted after spinal transection, showing that their reflex interference with the adrenergic discharge to the stomach can be excited by propriospinal connections

The effect of somatic depressor and pressor fibre stimulation on stomach motility

persisted after elimination of reflex movements in skeletal muscles by repeated administration of Flaxedil®

Discussion

In the present investigation which is one of a series of studies on the adrenergic control of stomach motility electric stimulation of somatic afferents was used for changing the flow of adrenergic activity to the stomach It is known that such afferents can either suppress or enhance the sympathetic discharge to the cardiovascular system with elicitation of depressor and pressor responses respectively (*cf* Johansson 1962) In analogy herewith the present study showed that the adrenergic supply affecting stomach motility can be suppressed by "somatic depressor fibres and enhanced by somatic pressor fibres resulting in opposite responses in the stomach because the adrenergic fibres *suppress* the motility of this effector organ The results are in agreement with the assumption of a pronounced antagonism between vagal cholinergic and splanchnic adrenergic effects on stomach motility and secondly with the hypothesis that the adrenergic nerves running to the stomach exert their primary action on ganglionic cells involved in the cholinergic control of stomach motility (Jansson and Martinson 1966 Jansson and Lisander 1969) It would thus appear that clear demonstration of adrenergic inhibition of stomach motility requires the existence of cholinergic nervous activity eliciting excitatory stomach motor responses This requirement is furthermore supported by the present study since the stomach inhibitory responses to somatic "pressor' afferent stimulation were significant only when cholinergic nervous activity was present either as a spontaneous background discharge in the vagal nerves or artificially induced by direct stimulation of the vagal excitatory fibres

The inhibition of stomach motility on somatic pressor afferent stimulation was prompt in onset with latencies as short as 4—5 sec The shortness of this latency argues strongly against a release of catecholamines from the adrenal glands being responsible for the primary action of the inhibition on pressor afferent stimulation (Fig 1) On the other hand a reflex release of catecholamines can very well explain the slight inhibition after a latency of 18—25 sec observed on somatic pressor stimulation in the absence of cholinergic excitatory motility of the stomach (Fig 1) The prompt inhibition of vagally induced excitatory motility on stimulation of somatic pressor afferents certainly indicates direct adrenergic mechanisms acting via splanchnic nerves which preferentially interact with stomach contractions dependent on cholinergic nervous activity

Demonstration of the effect of stimulation of "somatic depressor fibres on stomach motility required the existence of nervous activity in both the vagal cholinergic the splanchnic adrenergic nerve supply Concerning the nervous discharge in adrenergic nerves it is known that such experimental procedures as laparotomy abdominal surgery *per se* can initiate pronounced and sustained reflex activity of nerves and thereby inhibit contractions of the stomach which are in turn dependent on cholinergic nervous activity (*e g* Jansson and Lisander 1969) In the present ex

periments it was observed that vagally induced excitatory motility was more or less markedly inhibited during and after abdominal surgery (Fig 2) During this period stimulation of somatic afferents suppressed the background adrenergic activity and augmented vagally induced stomach contractions

In cats with transected spinal cords stimulation of somatic depressor afferents still had an augmentatory motor effect on vagally induced stomach motility This suggests that *these somatic afferents make spinal reflex connections with adrenergic* neurons affecting stomach motility But this does not exclude the possibility of an effect also on supra spinal centres involved in the adrenergic control of stomach motility

It is still not clear whether the adrenergic nerve effects to the stomach are involved mainly in patho-physiological events Group III muscle afferents mainly responsible for somatic depressor effects and for suppression of the flow of adrenergic activity to the stomach as described in this paper are thought to be involved in the nauseating type of deep pain A flow of reflex adrenergic activity from abdominal structures to the stomach can be induced but by stimulation which may be mainly nociceptive (Jansson and Martinson 1966 Jansson and Lisander 1969) The present investigation has shown that stimulation of somatic afferent pain fibres seems to produce similar adrenergic activity

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Acta physiol scand. 1969. 77. 23—27

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Catechol and Indole Derivatives in a Transplantable Islet-Cell Tumour of the Golden Hamster

By

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Abstract

CEGRELL, L, B FALCK and A M ROSENGREN *Catechol and indole derivatives in a transplantable islet cell tumour of the golden hamster* Acta physiol scand 1969 77 23—27

cells but no adrenergic nerves

An easily transplantable islet cell tumour in hamsters has recently been studied from histological and histochemical aspects (Grillo *et al* 1967) as well as from biochemical and metabolic aspects (Sodoyez *et al* 1967) This tumour has been shown to contain a much lower concentration of insulin than normal pancreatic tissue The tumour bearing animals develop a pronounced hypoglycaemia on fasting, in some animals a very high plasma level of immunoreactive insulin has been found (Sodoyez *et al* 1967)

A storage of biogenic monoamines in the endocrine pancreas is a common phenomenon among mammals, but in some species, *e g* the golden hamster, fluorescence histochemical investigations have so far failed to demonstrate any monoamines (see Cegrell 1968, p 14) The function of these monoamines and the significance of the species differences with respect to the occurrence of the monoam

are poorly understood Therefore, it was considered of interest to study the possible occurrence of catechol and indole derivatives in the islet cell tumour and in the pancreas of tumour bearing golden hamsters

Material and methods

The tumour[1] was transplanted subcutaneously (see Grillo *et al* 1967) to ten golden hamsters and allowed to grow for four to twelve weeks The animals were killed by decapitation and the tumours excised and analyzed chemically as described below Small pieces of the tumours and of the pancreatic glands were freeze-dried and processed for fluorescence microscopy

The estimation of catecholamines was made according to the procedure of Bertler *et al* (1958) as modified by Haggendal (1963) and that of 5 hydroxytryptamine as described by *Bertler (1961) In 4 expts dopa and dopamine were determined according to Anton and Sayre (1964) For further identification of catechol derivatives paper chromatographic analyses* were carried out in phenol—0 1 N HCl (9 1) on the eluates from the cation exchange column and on the Al O_3 eluates (*cf* Bertler *et al* 1958) Dopa and dopamine were used as reference substances To visualize the spots the chromatograms were sprayed with $K_3Fe(CN)_6$ or exposed to formaldehyde gas for 1 hr at +80° C.

In the histochemical studies the fluorescence method of Falck and Hillarp for the cellular localization of monoamines and some related compounds was used The freeze-dried tissues were exposed to formaldehyde gas for 1 hr at +80° C The specificity of the fluorescence was checked with the sodium borohydride reduction method of Corrodi *et al* (1964) Microscopic analyses of tissues not exposed to formaldehyde did not reveal any autofluorescence that could interfere with the observations of specific *i e* formaldehyde induced fluorescence Further details of the histochemical method have been given elsewhere (Falck and Owman 1965) Microspectrographic analyses of tissue sections and paper chromatograms were performed in a modified Leitz microspectrograph (*cf* Bjorklund *et al* 1968 a)

Results

The chemical analyses showed that the islet cell tumours contained substances that behaved like dopamine dopa and 5 hydroxytryptamine The concentrations of these substances are given in Table I When the methods of Bertler *et al* (1958) and Bertler (1961) were applied two substances were detected which showed the same elution and spectral characteristics as dopamine and 5 hydroxytryptamine respectively Noradrenaline or adrenaline could not be detected On the paper chromatograms of the eluates from the cation exchange column there appeared two fluorescent spots when the chromatograms were exposed to formaldehyde gas One of these spots was located as the authentic dopamine and displayed the typical catecholamine spectra when analyzed in the microspectrograph The other spot showed excitation/emission spectra with maxima at 400/430 mμ when the paper was exposed to NH_3 vapour for three minutes its intensity was highly increased Upon *spraying with* $K_3Fe(CN)_6$ only the dopamine spot appeared

With the method of Anton and Sayre (1964) substances behaving like dopa and dopamine were found in the tumour tissue The concentration of dopamine was approximately the same as in the above mentioned determinations No adrenaline or noradrenaline was detected On the paper chromatograms of the Al O_3 eluates two spots appeared with the same characteristics as authentic dopa and dopamine respectively In one experiment strips corresponding to these catechol spots were eluated with 0 01 N HCl and the fluorescence of the eluates (after oxidation and alkalinization) was read in an Aminco Bowman spectro photofluorimeter The spectral

[1] Two tumour-bearing animals were kindly placed at our disposal by Dr P Lefebvre Department of Medicine University of Liege Belgium

TABLE I The concentration of dopa dopamine (DA) and 5-hydroxytryptamine (5-HT) in an islet-cell tumour of the golden hamster Values are expressed as μg/g of fresh tissue Number of determinations within brackets

	Mean ± S E M (n)	Method used
Dopa	0 19 ± 0 09 (4)	Anton and Sayre (1964)
DA	2 62 ± 0 26 (4)	Anton and Sayre (1964)
DA	2 75 ± 0 36 (4)	Bertler *et al* (1958)
5-HT	0 87 ± 0 09 (5)	Bertler (1961)

characteristics were identical with those of authentic dopa and dopamine respectively

The fluorescence microscopy (Fig 1) revealed that the tumour cells emitted a moderate to sometimes intense green fluorescence which disappeared upon treatment of the sections in a sodium borohydride solution This fluorescence was absent in the specimens not exposed to formaldehyde No adrenergic nerves were found in the tumours Microspectrographic analyses demonstrated that the excitation/emission spectra of the cells showed maxima at 400/430 mμ In some experiments sections of the tumour were treated in HCl vapour according to the method of Björklund *et al* (1968 a) This allows a differentiation between the fluorophores of dopamine and noradrenaline During this treatment there was a marked reduction in the fluorescence intensity of the tumour cells but no certain shift in the excitation spectra

In the pancreatic islets a specific *i e* formaldehyde induced fluorescence was seen in adrenergic nerves forming peri and intra insular plexuses The nerves within the islets mostly followed the vessels and often formed a dense perivascular network No histochemically demonstrable amounts of biogenic monoamines were present in the islet cells

Discussion

It has recently been demonstrated that pancreatic islet cells in some mammals normally store biogenic monoamines Great species differences with respect to type and concentration of the monoamines however have been encountered and pronounced inter individual differences in their concentration have been recorded (*cf* Cegrell 1968) In some species *e g* the rat and golden hamster no histochemically demonstrable amounts of monoamines were found in the islet cells but this difference from other mammals can at least in the rat (*cf* Cegrell 1968) be a quantitative rather than a qualitative feature A study of the various aspects of these amines is necessary in order to understand their biological significance in hormone production. In the case of golden hamsters bearing this transplantable islet-cell tumour most of the circulating insulin seems to be secreted from the tumour the insulin secretion from the islets being inhibited (Lefebvre personal communication) The present study however did not reveal any histochemical difference between the pancreatic islets of normal and of tumour bearing hamsters *i e* there was no visible storage of monoamines in the cells the only specific fluorescence in the islets being

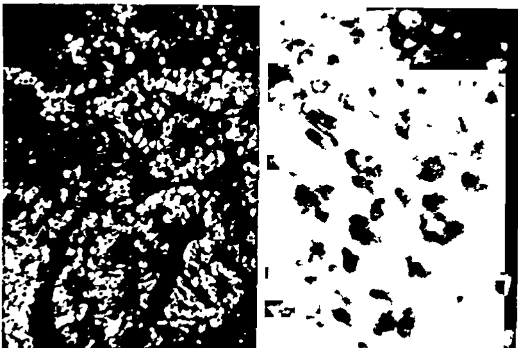


Fig 1 Formaldehyde-induced cytoplasmic fluorescence in cells of the islet cell tumour The cellular arrangement is similar that previously described by Grillo *et al* (1967) A 160×, B 360×

that emitted from numerous adrenergic nerve terminals intimately surrounding islet cells and vessels

In the tumour, on the other hand, a specific, *i e* formaldehyde-induced, cytoplasmic fluorescence was displayed by the cells and judged by the reaction conditions under which it developed and by the green colour of the emitted light in the fluorescence microscope (*cf* Falck and Owman 1965) it could derive from primary catecholamine or dopa However, the chemical analyses revealed also the presence of 5-hydroxytryptamine besides dopa and dopamine, and remarkably, an unknown substance capable of forming a fluorophore (on the paper chromatogram) upon treatment in formaldehyde gas (1 hr, +80° C) The identity of dopa and dopamine was established by the application of an absorption method and chromatographic methods whereas the presumption that 5-hydroxytryptamine too is present in the tumour tissue rests on the fact that a substance was demonstrated which behaved like 5-hydroxytryptamine on the ion exchange column The microspectrofluorimetric analyses revealed that the excitation/emission spectra of the tumour cells (400/430 mμ) clearly deviated from those of cells containing primary catecholamines (410/470 mμ) or 5-hydroxytryptamine (390/520—540 mμ) Further, the fluorescence intensity of the tumour cells markedly decreased upon HCl treatment of the sections, a phenomenon not seen in cells containing dopamine (Björklund *et al* 1968 a) or 5 hydroxytryptamine (unpublished observations) or in model systems (monoamine-containing albumin droplets Björklund *et al* 1968 b) Moreover, on the paper chromatograms of the eluates from the cation exchange

column there appeared upon exposure to formaldehyde gas two fluorescent spots One corresponded to dopamine, whereas the other was not located as dopa or dopamine and showed in the microspectrograph the same excitation and emission spectra as the tumour cells Upon alkalinization of the papers (developed in acid solution), the fluorescence intensity of this spot was highly increased which seems to agree with the finding that the fluorescence intensity of the tumour cells de creased upon acidification There is thus strong support for the view that the tumour cells contain an unknown substance whose formaldehyde induced fluorophore dominates in the recordings of the fluorescence spectra This substance does not seem to possess a free catechol nucleus since it did not show up on the paper chromatogram upon spraying with potassium ferricyanide it should be noted in this connection that it was not absorbed in Al_2O_3

Dopamine and 5 hydroxytryptamine are often found in the mammalian endocrine pancreas sometimes occurring simultaneously It is therefore of great interest that these two amines apparently exist together also in the islet cell tumour not least in view of the fact that the endocrine pancreas of the golden hamster does not store histochemically demonstrable amines either normally or when its insulin production is inhibited by an insulin secreting tumour

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From the Institute of Physiology, University of Oslo, Norway

Determination of Inulin, Albumin and Erythrocyte Spaces in the Bone Marrow of Rabbits

By

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Abstract

MICHELSEN, K *Determination of inulin, albumin and erythrocyte spaces in the bone marrow of rabbits* Acta physiol scand 1969 77 28—35

In 20 young rabbits weighing between 1 0 and 2 0 kg, inulin space, albumin space and erythrocyte space ... marrow of the femur by use of inulin-carboxyl ^{14}C ... Cr labelled autologous erythrocytes The mean ... ie mean albumin space 18 8 ml and the mean ery... that albumin passes the sinusoidal walls and is distributed throughout the extracellular space of the bone marrow

The bone marrow sinusoids, which are the exchange vessels in red bone marrow tissue differ from the exchange vessels in most other vascular beds both in structure and function In red bone marrow of rabbits the sinusoids are spindle-shaped or hexagonal structures with a diameter between 15 and 60 μ (Brånemark 1959) The sinusoidal walls are lined by reticuloendothelial cells which have cytoplasmic processes and which seem to form only a discontinuous layer (Zamboni and Pease 1961) Besides supplying blood to the bone marrow, the main function of the sinusoids seems to be to receive leucocytes and erythrocytes from the hematopoietic tissue

The structure of the bone marrow sinusoids has been thoroughly analysed but few studies have been performed on the micro-circulation and on the permeability properties of these peculiar vascular structures

In the present work the extracellular space albumin space and erythrocyte space of the bone marrow have been determined in the rabbit femur by simultaneous use of inulin-carboxyl-^{14}C ^{125}I labelled homologous albumin and ^{51}Cr labelled autologous erythrocytes The results indicate that the bone marrow vascular bed has a large volume and that albumin passes the sinusoidal walls quite freely and is distributed throughout the extracellular space of the tissue

Materials and methods

Preparation of the animals 20 young rabbits of both sexes and weighing between 1 0 and 2 0 kg were used They were anesthetized by i v injection of pentobarbitone (Nembutal®, Abbott), diluted in saline to a concentration of 12 mg per ml The dose was 30 to 40 mg per kg b w A catheter filled with heparinized saline was inserted into the right femoral artery for later blood sampling

Through dorsal incisions in the kidney regions the renal arteries, the renal veins and the ureters were ligated The kidneys were removed and the incisions closed by ligatures These procedures caused a blood loss which was estimated to be less than 5 ml

After removal of the kidneys a mixture containing ^{51}Cr-labelled autologous erythrocytes, ^{125}I labelled homologous albumin and inulin-carboxyl-^{14}C (the preparation of the mixture is described below) was injected through an ear vein The animals were then kept in light anesthesia by additional injection of 5 to 10 mg pentobarbitone per kg b w per hr After [illegible] (Heparin® A L) per kg b w i v , and [illegible] in the right femoral artery Then the [illegible])0 mg of pentobarbitone The shaft of [illegible] cutting the bone in the proximal and [illegible] middle part of the bone was removed [illegible] e bone marrow tissue cylinder in this [illegible] thick-walled glass tube for weighing [illegible] ass tube less than two min after the [illegible] iceps muscle was cut out and treated in the same way as the bone marrow sample for determination of albumin space in muscle

Preparation of ^{51}Cr labelled erythrocytes, ^{125}I-labelled albumin and inulin-carboxyl-^{14}C Before anesthesia, 6 ml of blood were taken from an ear vein of each animal Three ml of this sample were mixed with 1 0 ml of isotonic sodium citrate solution and centrifuged at 800 × g for 10 min The supernatant was removed and about 0 1 mC of $Na_2\,^{51}CrO_4$ was added to the erythrocytes [illegible]

sup[illegible]

T[illegible] non radio-

acti[illegible]

Separation and counting of the different radioisotopes in blood and tissue samples The blood taken from the animals immediately before they were killed was centrifuged at 3000 × g for 50 min About 0 1 ml of plasma was drawn into a pipette and transferred to a tube containing 1 0 g of non radio-active rabbit muscle tissue Non-radio active muscle was used to obtain approximately the same final volume of precipitated homogenate and the same final dilution of inulin carboxyl-^{14}C of the plasma sample as of the bone marrow sample The exact amount of plasma added to the tube was determined by weighing the pipette before and after the transfer of the plasma from the pipette to the tube

About 0 1 ml of packed erythrocytes was treated similarly and added to another tube also containing 1 g of non radio-active muscle tissue The samples of packed erythrocytes and of plasma then went through the same procedure as the marrow tissue sample

To the bone [illegible] 0 ml of 0 5 M trichloroacetic acid (TCA) 0 1 ml

0 1 [illegible] $AgCl$

T[illegible] d for 5 min by a glass pestle fitting the thick-

wall[illegible] e tube was centrifuged to obtain a clear super-

nata[illegible] would otherwise interfere with the scintillation

fluid 2 0 ml of the supernatant were immediately mixed with 1 0 ml of 0 5 M oxalic acid and centrifuged, whereby Ag^+ was precipitated as $Ag_2(COO)_2$ One ml of the supernatant ob-[illegible]

The precipitate obtained by centrifuging the tube with homogenized tissue contained practically all ^{125}I and ^{51}Cr of the bone marrow sample, either bound to precipitated protein or precipitated as AgI and Ag_2CrO_4 This precipitate was counted for ^{51}Cr and ^{125}I activity in a three Channel Auto gamma Spectrometer (Model 5022 Packard Instrument Comp),

By the gain and discriminator setting used, no ^{125}I activity was counted in the "window" set for ^{51}Cr counting. Some of the ^{51}Cr activity was, however, counted in the "window" set for ^{125}I counting. The "windows" were adjusted so that the ^{51}Cr emission gave a count rate in the "^{125}I window" which was 4 % of the count rate in the "^{51}Cr window". The counting time was adjusted so as to give at least 20,000 counts beyond back ground in each channel.

It was shown in preliminary experiments that about 15 % of the inulin carboxyl ^{14}C activity and about 5 % of the ^{125}I and $^{51}CrO_4$ activities were lost because of the procedures. With the doses of ^{125}I, $^{51}CrO_4$ and inulin carboxyl ^{14}C used, less than 5 % of the activity counted in the β liquid scintillation counter was due to ^{125}I and $^{51}CrO_4$ and more than 95 % to inulin carboxyl ^{14}C.

The hematocrit ratio of the arterial blood, sampled immediately before the killing of the animals, was determined using a micro capillary centrifuge (Model MB, International Equipment Company, Boston, Mass. U.S.A.)

Calculations and results

The erythrocyte space ($V_{Erythrocyte}$), the albumin space ($V_{Albumin}$) and the inulin space (V_{Inulin}) of the bone marrow were calculated from the following formulae

(I) $V_{Erythrocyte} = \frac{(^{51}Cr \text{ c p m /g) bone marrow}}{(^{51}Cr \text{ c p m /ml) packed erythrocytes}}$

(II) $V_{Albumin} = \frac{(^{125}I \text{ c p m /g) bone marrow}}{(^{125}I \text{ c p m /ml) plasma}}$

(III) $V_{Inulin} = \frac{(^{14}C \text{ c p m /g) bone marrow}}{(^{14}C \text{ c p m /ml) plasma}}$

(c p m = counts per min)

The volume of the samples of packed erythrocytes and of plasma were determined by dividing the weight of the samples by the specific gravity of packed erythrocytes (1.09) and of plasma (1.027) respectively. The volume of packed erythrocytes was ...d for trapped plasma.

The results of the individual experiments are given in Table I. A comparison between the inulin space and the albumin space shows that in all experiments the inulin space was somewhat larger than the albumin space.

Macroscopic examination revealed a red bone marrow tissue throughout the femoral bone marrow cavity in all animals.

Discussion

The purpose of the present study has been to obtain information about the size of the intravascular and interstitial compartments in bone marrow. Before discussing the results in detail it may be appropriate to consider the question whether the actual experimental conditions may have caused any marked disturbances in the physiological variables studied.

The animals were anesthetized by pentobarbitone. This drug has been found to cause a decrease in hematocrit ratio and in plasma protein concentration and an increase in the circulating blood volume in rats (Rieke and Everett 1957). Besides being anesthetized the rabbits were also nephrectomized in order to avoid excretion

TABLE I Bone marrow erythrocyte space ($V_{Erythrocyte}$), albumin space ($V_{Albumin}$), inulin space (V_{Inulin}) and hematocrit ratios of arterial blood (Hct_{art}) from 20 rabbits The arterial blood was sampled immediately before the death of the animals All space values are given in ml per 100 g tissue

Rabbit No	$V_{Erythrocyte}$	$V_{Albumin}$	V_{Inulin}	Hct_{art}
1	2 82	22 61	28 32	0 36
2	2 86	17 50	21 08	0 32
3	2 47	19 19	21 21	0 37
4	3 18	18 81	20 68	0 39
5	4 21	19 00	25 62	0 35
6	3 85	17 63	22.53	0 31
7	4 87	18.22	23 06	0.37
8	2 91	16.50	18 00	0 35
9	3 97	15 58	20 81	0 38
10	2 53	15 01	20 74	0 30
11	2 75	27.54	31 27	0 33
12	2 01	18 64	21 73	0 39
13	4.21	26 61	27 49	0 32
14	2 88	17 65	20 61	0 38
15	2 15	21 95	25 02	0.36
16	3 63	21 63	25 07	0 41
17	1 96	15 37	19 75	0 35
18	3 35	12 65	16 51	0 34
19	4 10	15 66	20 12	0 35
20	3 18	18 93	20 47	0 39
Mean	3 19	18 83*	22.50	0 36
S D	0 79	3 63	3.53	0 028

* The mean albumin space of quadriceps muscle, determined simultaneously in these experiments, was 0 91 ml per 100 g muscle (S D 0 26)

of inulin-carboxyl ^{14}C The blood loss was small and the animals seemed to stand this major surgical procedure well It should be noted however that Shires Williams and Brown (1961) using $^{35}SO_4$ to determine the extracellular space in man found that extensive surgical operations caused a decrease in the total extracellular fluid volume When evaluating the present findings it should be considered that the effects of pentobarbitone and nephrectomy in rabbits may be similar to those in rats and man

The time period chosen in the present experiments to obtain an adequate mixing of the labelled inulin, albumin and erythrocytes in the rabbits was about 2 5 hrs Within this period, inulin will reach an approximate diffusion equilibrium in the extracellular space (Law and Phelps 1966) In most vascular beds much less time is needed to obtain a complete mixing of erythrocytes and albumin In the

marrow however 2 5 hrs might have been a somewhat short equilibration time for albumin for reasons to be discussed below In spite of this 2 5 hrs were chosen to make the possible long term effects of nephrectomy and pentobarbitone anesthesia as small as possible

Like any substance applied for determination of the extracellular space inulin carboxyl ^{14}C can also be criticized Several authors (Nichols et al 1953 Cotlove 1954) have thus maintained that the inulin space when measured 2 5 hrs after inulin injection is less than the true extracellular space because of slow permeation of inulin into the interstitial space of poorly vascularized collagenous structures However red bone marrow tissue is richly vascularized and seems to lack collagenous structures Inulin should therefore be well suited for determination of the extra cellular space in bone marrow It may be mentioned that Chen Terepka and Lane (1963) have found that inulin carboxyl ^{14}C differs in some physico chemical and physiological properties from unlabelled inulin but space determinations performed with the two indicators agree satisfactorily (Dow and Irvine 1967 Addanki Cahill and Sotos 1967 Wilde 1945 Law and Phelps 1966)

The mean space occupied by erythrocytes from the circulating blood was 3 2 ml per 100 g bone marrow in the present group of growing rabbits which all had com pletely red femoral bone marrow tissues as judged from macroscopic examination Brookes (1965) found 1 6 ml of circulating erythrocytes per 100 g tissue in the femoral bone marrow of rats and Osmond and Everett (1965) found 2 7 ml per 100 g bone marrow in the femur of guinea pigs The smaller erythrocyte space found by these investigators particularly by Brookes may be due to species and age dif ferences However it should be noted that in the present experiments 2 5 hrs elapsed from the injection of the labelled erythrocytes to the death of the animal whereas in the experiments of Brookes and of Osmond and Everett that period was 15 min Also Osmond and Everett found that the calculated bone marrow erythrocyte space of guinea pigs increased 2 to 3 times when the equilibration period was in creased from 15 min to 3 hrs These authors using ^{59}Fe labelled homologous erythrocytes suggested that this increase indicated a selective trapping of erythro cytes in the bone marrow The labelled erythrocytes used in the present experi ments were autologous Provided therefore that the labelling procedure had not changed the cells there should be no particular tendency for them to be selective ly trapped in the bone marrow

The inulin space found for bone marrow in the present experiments (mean 22 5 ml per 100 g bone marow) is greater than that of skeletal muscle which appears to be between 10 and 15 ml per 100 g tissue (Wilde 1945 Addanki *et al* 1967) The present value for bone marrow is of the same magnitude as the inulin space reported for heart muscle (Spach 1965 Addanki *et al* 1967) liver (Addanki *et al* 1967) and for the body as a whole (Spach 1965) It thus appears that the extracellular space of the soft blood cell producing bone marrow tissue does not differ greatly from that of many other tissues which are much less proliferous

A comparison between inulin space and albumin space in bone marrow shows

that the albumin space is about 84 per cent of the inulin space The great value found for the albumin space was apparently not due to abnormal properties of the [131]I labelled homologous albumin preparation used since the skeletal muscle albumin space was found to be 0 91 ml per 100 g tissue It may also be mentioned that the bone marrow albumin space determined in preliminary experiments on unanesthetized rabbits with intact kidneys was of the same magnitude (17 5 ml per 100 g) as in the anesthetized nephrectomized animals of this study The great size of the albumin space both in absolute terms and in relation to the inulin space strongly suggests that the sinusoidal walls are permeable to albumin and that albumin and inulin are dispersed within practically the same anatomical compartments If the bone marrow sinusoids were impermeable to albumin then the size of the albumin space would imply that the mean volume of the bone marrow vascular bed ($V_{Erythrocyte}+V_{Albumin}$) would be 22 0 ml per 100 g tissue, which is enormous Furthermore the mean bone marrow interstitial space ($V_{Inulin}-V_{Albumin}$) would be only 3 7 ml per 100 g tissue which is exceptionally small and finally the hematocrit ratio for the intravascular blood in the bone marrow ($V_{Erythrocyte}/(V_{Erythrocyte}+V_{Albumin})$) would be as low as 0 15

Since the bone marrow sinusoids thus seem to be permeable to albumin the volume of the bone marrow vascular bed can be calculated only if the hematocrit ratio of the intravascular blood is known This ratio cannot be obtained from the present results but if it is assumed that the hematocrit ratio of the blood within the bone marrow vascular bed equals that of the arterial blood (0 36) the mean volume of the intravascular plasma space would be 5 7 ml per 100 g tissue The mean volume of the bone marrow vascular bed would then be 8 9 ml per 100 g tissue and the mean interstitial space 16 8 ml per 100 g tissue In this connection it should be noted that the hematocrit ratio of the blood in most tissues is lower than that of the arterial blood (Dewey 1960) If this is so also in the bone marrow the calculated value for the intravascular volume is too low On the other hand if the bone marrow has an erythrocyte reservoir function similar to that of the spleen the value is too high

The small difference between the albumin and the inulin space can possibly be explained as a result of the difference in molecular size between albumin (molecular weight 70 000) and inulin (molecular weight 5500) It has been shown by Law and Phelps (1966) and by Tasker *et al* (1959) that the distribution volume of several substances used to determine the extracellular space decreases with increasing molecular weight of the substances Another possibility is that complete diffusion equilibrium had not been reached for the large albumin molecules within the 2 5 hrs period from the injection of labelled albumin to the death of the animals Even though albumin might not have reached diffusion equilibrium the size of the albumin space reached in the course of 2 5 hrs strongly suggests that the bone marrow sinusoids are quite permeable to albumin However a high permeability of the bone marrow exchange vessels to albumin does not necessarily result in a great value for bone marrow albumin space As shown by Dewey (1959) the protein permeability

of the vascular bed of several viscera is high and, in spite of this the plasma protein concentration in the tissues of these organs is small Dewey explained this as a result of an efficient lymphatic drainage of plasma proteins Thus, if the lymphatic elimination of proteins is large, the extracellular plasma protein concentration can be low, even if the vascular bed is very permeable to proteins The present observations on bone marrow indicate that the lymphatic drainage of albumin from this tissue must be small compared with the passage of albumin through the sinusoidal walls This agrees well with findings indicating that the bone marrow is not supplied with lymph vessels (Yoffey and Courtice 1956 p 12)

It would be of interest to know whether the bone marrow interstitial fluid which apparently contains albumin in concentrations close to that in plasma also contains significant amounts of the larger plasma proteins, especially globulins The present experiments give no information on this point However, if the bone marrow lacks lymphatic drainage and does not metabolize plasma proteins rapidly, even a moderate sinusoidal permeability to a protein would cause it to appear in the interstitial fluid *in about the same concentration as in plasma*

The high protein content of interstitial fluid in bone marrow implies that the colloid osmotic pressure difference across the sinusoidal walls must be small or absent According to the Starling hypothesis for transvascular fluid filtration and absorption, the hydrostatic pressure difference across the sinusoidal walls must then also be small or absent This does indeed seem to be the case In an analysis of the pressure relationships in the bone marrow vascular bed (Michelsen 1967) it was concluded that the tissue pressure and the intravascular pressure past the arterioles are about equal

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The Evolution of Vascular Smooth Muscle Responses to Histamine and 5-Hydroxytryptamine

II. Appearance of inhibitory actions of 5-hydroxytryptamine in amphibians[1]

By

OLA BODVAR REITE[2]

Received 20 November 1968

Abstract

REITE, O B *The evolution of vascular smooth muscle responses to histamine and 5-hydroxytryptamine II Appearance of inhibitory actions of 5-hydroxytrypta-mine in amphibians* Acta physiol scand 1969 77 36—51

Systematic studies on the vascular smooth muscle responses to histamine and 5 hydroxytrypta mine, similar to those previously accomplished in jawless vertebrates and fish were performed in amphibians (frogs toads and salamanders) Both intact animals and artificially perfused preparations were studied The effects were recorded as changes in systemic arterial blood pressure after intravascular administration of drugs or as changes in vascular resistance after introduction of drugs into the perfusion fluid during perfusion at constant flow rate Responses to adrenaline noradrenaline and acetylcholine were studied for comparison The direct or .. nature of actions produced by the different drugs were evaluated by means of pharma al blocking agents The outcome of the studies may be summarized as follows Marked specific inhibitory actions of 5 hydroxytryptamine on vascular smooth muscle, manifest vasodilatation are present in all studied species displaying a feature which has not been emonstrated in jawless vertebrates and fish In addition the blood vessels of some amphibians occasionally show a constrictor response to 5 hydroxytryptamine The nature of the latter response remains uncertain Histamine apparently has but negligible influence on amphibian vascular smooth muscle The inhibitory and stimulatory actions of adrenaline and noradrenaline and the stimulatory action of acetylcholine, pharmacological characteristics of vascular smooth muscle also in more primitive vertebrates are retained in the amphibians while the inhibitory action of acetylcholine like the inhibitory action of 5 hydroxytryptamine, appears as a new feature

A previous report from an investigation on evolutionary changes in the actions of histamine and 5 hydroxytryptamine on vertebrate vascular smooth muscle dealt with results obtained in jawless vertebrates and fish (Reite 1969a) Wherever specific actions of either drug were found their nature was stimulatory As will be revealed by

[1] Partial reports from this work were presented at the Fall Meeting of the American Physiological Society, Los Angeles August 1965, and at the Annual General Meeting of Stockholm Physiological Society, Stockholm November 1967

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the present work the picture becomes different when the experiments are extended to amphibians In this class of vertebrates the presence of inhibitory actions of 5 hydroxytryptamine seems to be a general feature

Observations were made in several species of frogs, toads and salamanders The experimental approach was principally similar to that applied in fish Arterial blood pressure changes in intact animals, and resistance changes in artificially perfused preparations were recorded following administration of histamine, 5 hydroxytryptamine and agents which release endogenous stores of these amines and the effects were compared with the effects of adrenaline, noradrenaline and acetylcholine The specificity of the action of different drugs on vascular smooth muscle was studied by using pharmacological blocking agents Additional information was gained by chemical assay of biogenic amines in tissues or perfusates and by histochemical localization of 5 hydroxytryptamine

Materials and methods

Experimental animals The studied species are listed in Table 1 They were acquired from commercial sources and kept without food in lucite boxes in temperature-controlled rooms, the tropical species *(Bufo marinus)* at 18—22° C and those from temperate regions usually at 4—6° C However, some specimens from the latter species were also kept at ambient temperatures of 18—22° C until being used or transferred from the cold room to this temperature 2—3 weeks prior to use The boxes for housing of frogs and mudpuppies contained some water but the frogs had access to bricks rising above the water surface Toads and salamanders were free to choose between staying in moss or in water The water was [illegible]

cannulated in central direction for administration of pharmacological agents Prior to the cannulations both catheters had been connected to three way stop-cocks and filled with heparinized physiological solution Anesthetization and implantation of arterial and venous [illegible]

doses [illegible]
(throi [illegible]
servat [illegible]

[illegible] up [illegible] iade [illegible] 1 to

water with the indwelling catheters intact. When recovery from anesthesia seemed complete, the effects of drugs on the blood pressure of these frogs were recorded in the same way as in anesthetized specimens Such experiments took place within the first few hours of implantation of catheters The studied animals were always sacrificed upon completion of the experi

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Abstract

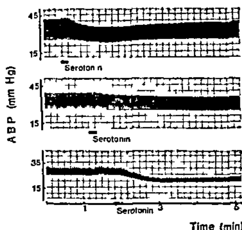
REITE, O B *The evolution of vascular smooth muscle responses to histamine and 5-hydroxytryptamine II Appearance of inhibitory actions of 5-hydroxytrypta mine in amphibians* Acta physiol scand 1969 77 36—51

Systematic studies on the vascular smooth muscle responses to histamine and 5 hydroxytrypta mine, similar to those previously accomplished in jawless vertebrates and fish, were performed in amphibians (frogs toads and salamanders) Both intact animals and artificially perfused preparations were studied The effects were recorded as changes in systemic arterial blood pressure after intravascular administration of drugs or as changes in vascular resistance after introduction of drugs into the perfusion fluid during perfusion at constant flow rate Responses to adrenaline noradrenaline and acetylcholine were studied for comparison The direct or indirect nature of actions produced by the different drugs were evaluated by means of pharma cal blocking agents The outcome of the studies may be summarized as follows Marked specific inhibitory actions of 5 hydroxytryptamine on vascular smooth muscle manifest vasodilatation are present in all studied species displaying a feature which has not been demonstrated in jawless vertebrates and fish In addition the blood vessels of some amphibians occasionally show a constrictor response to 5 hydroxytryptamine The nature of the latter response remains uncertain Histamine apparently has but negligible influence on amphibian vascular smooth muscle The inhibitory and stimulatory actions of adrenaline and noradrenaline and the stimulatory action of acetylcholine pharmacological characteristics of vascular smooth muscle also in more primitive vertebrates are retained in the amphibians while the inhibitory action of acetylcholine like the inhibitory action of 5 hydroxytryptamine appears as a new feature

A previous report from an investigation on evolutionary changes in the actions of histamine and 5 hydroxytryptamine on vertebrate vascular smooth muscle dealt with results obtained in jawless vertebrates and fish (Reite 1969a) Wherever specific ac tions of either drug were found their nature was stimulatory As will be revealed by

[1] Partial reports from this work were presented at the Fall Meeting of the American Physio logical Society Los Angeles August 1965 and at the Annual General Meeting of Stockholm Physiological Society, Stockholm November 1967

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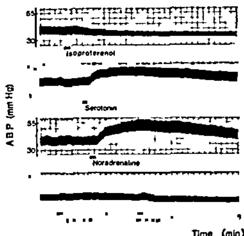


Fig 1 Fig 2

Fig 1 Effects of 5 hydroxytryptamine (serotonin) on aortic blood pressure (A B P) in the American bullfrog (upper tracing) the marine toad (middle tracing) and the mudpuppy (lower tracing) The drug was administered intravenously, 5 μg to the American bullfrog and 2 μg to each of the other species The animals were anesthetized with tricaine methane sulfonate and the experiments performed at 18° C.

Fig 2 Aortic blood pressure changes following intravenous injections of isoproterenol (4 μg), 5 hydroxytryptamine (serotonin 3 μg), noradrenaline (4 μg), histamine (30 μg) and 5 hydroxytryptamine (serotonin, 3 μg) in the common toad anesthetized with urethane, at 22° C. The three upper tracings were obtained early during the experiment the lower tracing after the experiment had lasted for about 1 hr Note reversal of the response to 5 hydroxytryptamine

spectrofluorometrically after adsorption onto aluminium oxide and oxidation of the subsequent eluate to a fluorescent trihydroxyindole derivative according to the method of Anton and Sayre (1962)

observations were made with a Leitz Ortholux microscope equipped for fluorescence microscopy with camera attached

Results

Initial heart rate and blood pressure

In all studied species both heart rate and blood pressure became stable within a few minutes of completion of the surgical procedure Under this condition prior to administration of any drug the heart rates most commonly encountered were 30—60 beats per minute at temperatures of 18—22° C and 15—20 beats per minute at 10—12° C Systolic arterial blood pressure at 18—22° C was usually in the range of 30—45 mm Hg while the corresponding diastolic pressure was 20—32 mm Hg In experiments performed at 10—12° C, the average blood pressure was 5—10 mm Hg below that obtained at the higher temperatures

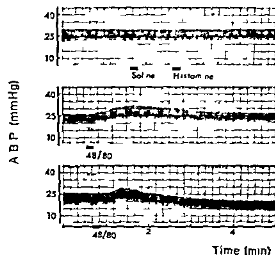


Fig 3 Effects of successive intravenous [illegible] blood pressure of the leopard frog The experiment was performed under urethane anesthesia at 18° C

Blood pressure effects of pharmacological agents

Intravenous injections of histamine in doses of 5—200 μg did not significantly affect either heart rate or blood pressure in any species Occasionally, a slight increase in pulse pressure and pressure level was noted, but this increase was similar to that obtained after control injection of an equal volume of physiological solution, and must be ascribed to increased venous return Intra-arterially administered histamine also [illegible] no effect

ntrary to the absence of blood pressure responses to histamine 5-hydroxytryptamine (1—20 μg) consistently produced marked effects A decrease in blood pressure, occasionally preceded by a slight and transient increase was usually observed (Fig 1) The response pattern found in the common toad was the only exception In some specimens from this species studied during early summer the first few injections of 5-hydroxytryptamine performed at the beginning of an experiment resulted in a pressure increase while subsequent injections showed a depressor action (Fig 2)

Compound 48/80 elicited changes in blood pressure in all studied species In frogs, the response to the first 2—3 of several successively administered small doses (10—20 μg) was a pressure increase (Fig 3) The subsequent injections of such doses either gave no response or a slight decrease in blood pressure High doses (50—150 μg) produced a transient pressure increase followed by a long-lasting decrease Repeated injections of high doses led to a continuous fall in blood pressure and normal pressure levels did not reappear Polymyxin B (150—2000 units) produced blood pressure changes similar to those produced by compound 48/80, and the effects of both agents were qualitatively the same in all species of frogs In toads and salamanders, compound 48/80 and polymyxin B mimicked the actions of 5-hydroxytryptamine (Fig 4)

Adrenaline and noradrenaline (2—20 μg) had marked pressor effects in all studied species of frogs and salamanders, but less marked and less persistent pressor effects in

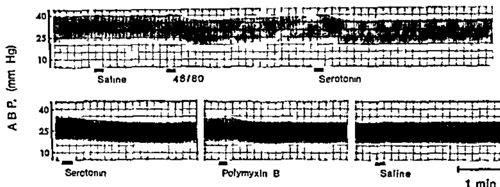


Fig 4 Aortic blood pressure recording in urethane anesthetized marine toads at 18° C The upper tracing shows the effects of successive intravenous injections of physiological solution (saline), compound 48/80 (30 μg) and 5 hydroxytryptamine (serotonin 2 μg) in one toad while the lower tracings show the effects in another toad of similarly injected 5-hydroxytryptamine (serotonin, 2 μg), polymyxin B (500 units) and physiolocigal solution (saline) Note the absence of response to control injections of physiological solution and the striking similarities in the responses to compound 48/80 and polymyxin B and the response to 5 hydroxytryptamine

toads In marine toads, it was observed that in the late part of an experiment, when the animals had often received total amounts of histamine of 200—500 μg the pressor effect of adrenaline and noradrenaline was abolished or reversed During this condition, doses as high as 100 μg of either catecholamine failed to raise the blood pressure Isoproterenol (1—5 μg) always produced an increase in heart rate and a decrease in blood pressure (Fig 2) Bradykinin (5—10 μg) had the effect of reducing blood pressure, but this drug was tested only in the European common frog and the European edible frog Acetylcholine (0 1—5 μg) usually caused a slowing of the heart, but the lowest doses, especially when administered intra-arterially, sometimes elicited a decrease in blood pressure without any apparent effect on heart rate

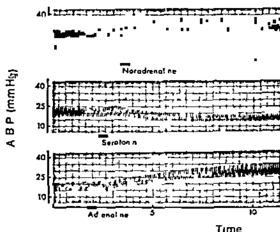


Fig 5 Effects of noradrenaline (3 μg), 5 hydroxytryptamine (serotonin, 5 μg) and adrenaline (5 μg) on the aortic blood pressure of the American bullfrog anesthetized with tricaine methanesulfonate, at 10° C At this temperature the duration of action of all drugs is prolonged, but otherwise the effects are principally similar to those obtained at higher temperatures

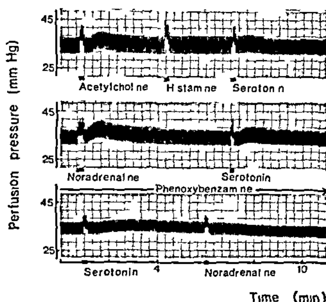


Fig 6 Perfusion of the hind part of the ribbed salamander The two upper tracings show changes in perfusion pressure following successive injections of single doses of acetylcholine (15 μg) histamine (40 μg) 5 hydroxytryptamine (serotonin 10 μg) noradrenaline (5 μg) and 5 hydroxytryptamine (serotonin 10 μg) into the perfusion circuit (between the pump and the vascular bed) The marked and transient pressure rise associated with each injection is due to the injected fluid volume and is evident in all tracings from perfusion experiments Effects of drugs on vascular resistance appear as slower changes in perfusion pressure Note that the first injection of 5 hydroxytryptamine produces a dual response whereas the second injection of this drug produces only a pressure in

Differences in the temperature at which the experiments were performed did not the nature of the response to any drug (Fig 5) neither did differences in ambient temperature at which the animals had been housed prior to the experiments

The influence of pharmacological blocking agents on the different blood pressure responses was studied in three species the European common frog the European edible frog and the common toad This showed that intravascular administration of methysergide in doses of 50—200 μg diminished or completely abolished the depressor response to 5 hydroxytryptamine The pressor response to 5 hydroxytryptamine in the common toad was too inconsistent to allow evaluation of specificity by use of blocking agents Atropine (40—100 μg) and the β adrenergic blocking agent propranolol (40—100 μg) abolished the cardiovascular responses to acetylcholine and isoproterenol respectively whereas similar doses of the α adrenergic blocking agents phentolamine or phenoxybenzamine abolished the pressor response to adrenaline and noradrenaline Apart from methysergide none of the tested agents influenced the depressor response to 5 hydroxytryptamine

Effects obtained in perfused preparations

In all studied species histamine administered as single doses of 10—200 μg into the perfusion circuit produced negligible effects (Fig 6 and 7) Continuous infusion of histamine (2—5 μg/ml) similarly failed to change the perfusion pressure significantly

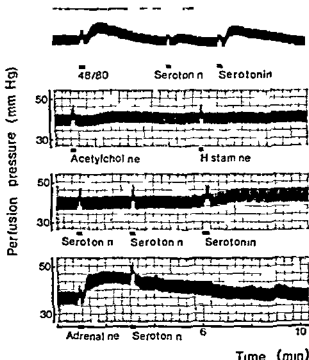


Fig 7 Perfusion experiments in common toads illustrating the marked individual variations in sensitivity to 5 hydroxytryptamine The two upper tracings demonstrate the effects of compound 48/80 (15 µg) 5-hydroxytryptamine (serotonin 2 µg and 10 µg), acetylcholine (10 µg) and histamine (30 µg) injected into the perfusion circuit during one experiment The two lower tracings were obtained in an other preparation in which successive injections of 5 hydroxytryptamine (serotonin 5 µg 10 µg and 100 µg) failed to elicit a clear pressor response except at the highest dose level and where the effect of 5 hydroxytryptamine (serotonin 10 µg) was also tested and found to be negligible after the perfusion pressure had been raised by a previous injection of adrenaline (15 µg)

The effects of 5 hydroxytryptamine (2—150 µg) were also weak in most species Injections performed shortly after start of perfusion occasionally caused a small decrease in perfusion pressure but the pressure did not return to the initial level (Fig 6) The only effect obtained after repeated injections was an increase in perfusion pressure The sensitivity was highly variable and usually low However early during some experiments in European common frogs and common toads 5 hydroxytryptamine produced a strong pressure increase whereas the effect of subsequent injections was less marked (Fig 7 and 8) Continuous infusion of 5 hydroxytryptamine (2—5 µg/ml) produced only slight changes in perfusion pressure After administration of 5 hydroxytryptamine visible rhythmic contractions appeared in the gut These contractions often continued for several minutes and sometimes even disturbed the perfusion pressure recording mechanically

The responses to compound 48/80 (10—100 µg) and polymyxin B (150—1000 units) were similar and reference will therefore only be made to the actions of compound 48/80 Low doses (10—15 µg) administered at the beginning of an experiment produced an increase in perfusion pressure in frogs (Fig 9) and occasionally in the common toad (Fig 7) but tachyphylaxis soon developed Higher doses (20—100 µg) induced a long lasting increase in perfusion pressure in all species This response was most conspicuous in the European common frog in which continuous infusion of compound 48/80 (0 2—1 µg/ml) was also tried Such infusion resulted in a rapid increase in perfusion pressure followed first by a transient decline

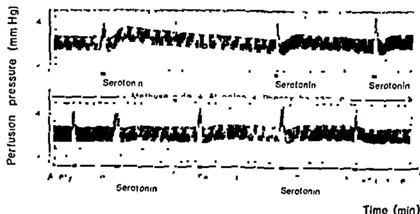


Fig 8 Perfusion experiment in the European common frog The upper tracing, obtained early during the experiment, demonstrates the decreasing response to successive injections of 5-hydroxytryptamine (serotonin, 10 μg, 10 μg and 15 μg) The lower tracing shows that weak pressor responses to 5-hydroxytryptamine (serotonin, 15 μg, 20 μg and 25 μg) persist after *addition of methysergide (8 μg/ml), atropine (3 μg/ml) and phenoxybenzamine (3 μg/ml) to* the perfusion fluid, whereas the normal pressor response to acetylcholine (10 μg) is abolished and the normal pressor response to noradrenaline (5 μg) is reversed

and then by a steady increase Finally, the perfusion pressure became stabilized at a level 3—4 times higher than the initial level As long as the infusion of compound 48/80 was *continued, the high perfusion pressure could be maintained, but it gradual*ly decreased to close to the initial level when perfusion with pure physiological solution was re-established If the experiment was repeated, the first rapid increase in erfusion pressure did not reappear However, the slower and steady increase to a gh pressure level was still present

Adrenaline and noradrenaline (2—20 μg) consistently increased the perfusion ressure (*Fig 6, 7 and 9) The perfusion pressure was also increased by 5—20 μg of* acetylcholine (Fig 6 and 7), but early during an experiment a weak depressor effect of acetylcholine was occasionally observed after administration of low doses Isoproterenol (5—10 μg) produced a slight decrease in perfusion pressure, while bradykinin (10—20 μg), which was tested in the European common frog and the European edible frog, produced a more marked decrease

The influence of pharmacological blocking agents was thoroughly studied only in preparations from the European common frog the common toad and the ribbed salamander The pressor effect of adrenaline and noradrenaline was reversed or abolished by dihydroergotamine (2—5 μg/ml), phenoxybenzamine (2—5 μg/ml) or phentolamine (4 μg/ml) while the weak pressor effect of 5-hydroxytryptamine remained (Fig 6 and 8) Blocking of the depressor effect of adrenaline and noradrenaline (evident after *α-adrenergic receptor blockade) could be achieved with the β-*adrenergic blocking agent propranolol (3 μg/ml), which also abolished the effect of isoproterenol The effect of acetylcholine was blocked by atropine (3 μg/ml), and this blockade apparently did not change the responses to any of the other drugs Since the strong pressor responses to 5-hydroxytryptamine and low doses of com-

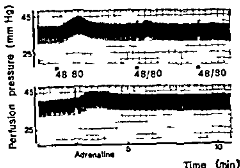


Fig 9 Development of tachyphylaxis to low doses of compound 48/80 in the perfused hind legs of the American bullfrog After the first of three successive injections of equal doses (15 μg) of this agent there is a marked pressor response after the second a weak pressor response and after the third no response (upper tracing) The preparation still shows a normal response to adrenaline (5 μg lower tracing)

pound 48/80 were rather inconsistent evaluation of their specificity by means of blocking agents could not be made with certainty However, in the European common frog it was found that such strong responses to compound 48/80 were also present in experiments where methysergide (5—10 μg/ml) had been added to the perfusion fluid Similarly the weak pressor responses to 5 hydroxytryptamine and high doses of compound 48/80 which often persisted throughout an experiment were apparently unaffected by methysergide (Fig 8) The slow component in the pressor response obtained during continuous infusion of compound 48/80 could be blocked by phenoxybenzamine or dihydroergotamine in concentrations of 2—5 μg/ml Methysergide and dihydroergotamine commonly elicited visible rhythmic contractions of the gut similar to those produced by 5 hydroxytryptamine

Tissue levels of biogenic amines

Histamine and 5 hydroxytryptamine were assayed in skin muscle lung and stomach from the European common frog in skin and lung from the European edible frog and the common toad and in skin and stomach from the fire salamander and the ribbed salamander The samples of skin from frogs and toads consisted of the entire skin of the hind legs while those from salamanders were taken from the tail In all studied tissues except the stomach the histamine levels were in the range between 0 05 μg/g and 0 5 μg/g Determinations in 24 European common frogs 3 fire salamanders and 3 ribbed salamanders showed the histamine content of whole stomach of these species to be 5 5—12 1 μg/g 4 7—6 3 μg/g and 3 6—5 9 μg/g respectively The histamine stores in the stomach of the European common frog were apparently also maintained in specimens kept at temperatures of 4—6° C without food for several months and application of indicator paper to the surface of the gastric mucosa of a few of the frogs taken from the cold room showed a pH below 3

Two tissues the skin and the stomach from the ribbed salamander showed absence of 5 hydroxytryptamine (levels below 0 2 μg/g) while all studied tissues except skin from the other species contained small amounts (usually 0 2—0 6 μg/g but occasionally up to 1 5 μg/g) High levels of 5 hydroxytryptamine were encountered in skin from the common toad (47—94 μg/g) the spotted salamander (35—62 μg/g)

and the European edible frog (53—195 µg/g), which were all studied during the autumn and winter, and also in skin from the European common frog. In the latter species the 5-hydroxytryptamine content of skin was determined in some 60 males and a few females killed at different seasons of the year. There seemed to be large seasonal variations, and also considerable individual variations within each season. The values showed a maximum during the summer, and a minimum during the winter. Thus in January—February, the range was 35—218 µg/g, and during July and August, 342—876 µg/g. The 5-hydroxytryptamine levels in the few female specimens studied showed no marked difference from those of the males. Seasonal changes were evident both in frogs kept in the cold room (4—6° C) and in frogs killed and subjected to assay immediately after being captured in their natural environment and taken to the laboratory.

Determinations were also made of the 5-hydroxytryptamine content in skin of European common frogs previously treated with compound 48/80. Since individual variations in normal frogs were very large, the interpretation of the results cannot be conclusive. However, no evidence was found of any high degree of depletion. This applies both to frogs which received a single injection of compound 48/80 (400—500 µg) into the dorsal lymph sac and to frogs similarly treated over four days with daily injections of lower but increasing doses (50, 75, 125 and 200 µg). The injection of compound 48/80 at high dosage (400—500 µg) resulted in stimulation or emptying of cutaneous glands, and in marked lightening of the skin. The secretion from the skin was so abundant that if a few of the injected frogs were placed in a small jar they became entirely covered with a foamy layer (Fig. 10). After pouring water into this jar containing the frogs (10 ml per frog), the 5-hydroxytryptamine content of water samples withdrawn during the first half an hour of injection of compound 48/80 was determined and found to be 10—30 µg/ml. Assay for catecholamines in these samples showed no noradrenaline, but occasionally there was evidence of the presence of very small amounts of adrenaline. Similar water samples taken from a jar containing frogs treated with control injections of physiological solution showed a 5-hydroxytryptamine content below 2 µg/ml. The studies on 5-hydroxytryptamine in skin secretions were performed during the summer. Assays of histamine in whole stomach of frogs treated with compound 48/80 indicated that the levels of this amine were unaltered.

Apart from the changes described above, the general condition of European common frogs treated with compound 48/80 was severely affected. After an injection of 400—500 µg, dilatation of small blood vessels was evident in all parts of the skin where pigmentation did not prevent such observations being made, and muscular movements usually ceased within an hour. Frogs receiving this high dose never recovered. Daily injections of increasing doses (50, 75, 125 and 200 µg) were also poorly tolerated. After only 2—3 such injections, the frogs started to imbibe water as indicated by swelling and weight increase. High doses of 5-hydroxytryptamine (1—2 mg) produced no conspicuous ill effects, but administration of this drug was followed by defecation and increased secretion from the skin.



Fig 10



Fig 11

Fig 10 Male European common frogs five in either jar The frogs in the right hand jar have each received an injection of compound 48/80 (400 µg) dissolved in 1 ml of physiological solution while those in the left hand jar have received control injections of the same volume of physiological solution The photograph was obtained about half an hour after the injection and indicates that cutaneous secretion is greatly enhanced by compound 48/80

Fig 11 Frog skin treated with formaldehyde gas as seen through the fluorescence microscope Cutaneous glands showed a bright yellow fluorescence Magnification 65×

The effect on the skin 5 hydroxytryptamine content from perfusion of the hind legs of the European common frog with compound 48/80 was also studied in a few specimens In this case, the skin of one leg was removed for assay before starting the perfusion After perfusion for 30—60 min with a concentration of compound 48/80 of 1 µg/ml the total 5 hydroxytryptamine content in the skin of the remaining leg was reduced to about 70 % of that of the control Measurable quantities of 5 hydroxytryptamine could be recovered from the effluent perfusion fluid and evidence was also found of the presence of low levels of adrenaline

Histochemical localization of 5 hydroxytryptamine in skin

A faint and diffuse yellow green fluorescence was observed throughout the studied skin samples from the European common frog However distinct and strong yellow fluorescence was present only in cutaneous glands (Fig 11) Both the secretory cells and the secretions present in the duct leading to the surface of the skin seemed to be fluorescing The yellow fluorescence was absent in untreated control preparations After subsequent staining with 0 1 % alcoholic thionin numerous tissue mast cells indiscernible in the fluorescence microscope were easily identified by their metachromatic staining reaction The mast cells were located among the blood vessels in the richly vascularized subcutaneous connective tissue layer

Discussion

The results from all studied species of amphibians indicate that presence of both inhibitory and stimulatory actions of adrenaline and noradrenaline on vascular smooth muscle is a general characteristic within this class of vertebrates as it is in jawless vertebrates and fish (Reite 1969a) Stimulatory actions of acetylcholine which are also present in more primitive vertebrates are similarly retained while the inhibitory action of acetylcholine on vascular smooth muscle seems to be a new feature Since none of the studied preparations failed to respond to adrenaline nor adrenaline and acetylcholine the experimental design should be adequate to reveal the actions of histamine and 5 hydroxytryptamine as well

Early studies reviewed by Feldberg and Schilf (1930) demonstrated an extremely low sensitivity to histamine in the vascular system of frogs According to the present results this apparently also applies to toads and salamanders There is some evidence that perfused blood vessels from frogs may occasionally show weak constriction after administration of histamine especially during the winter whereas there may be slight dilatation during the summer (Martin and Lissak 1940 Kadatz 1949—50 Heim and Meves 1950) However both the present results nd information made available through previous work (Grant and Jones 1927—29 Krogh 1929 Feldberg and Schilf 1930 Reite 1965) indicate that the histamine dose necessary to elicit any vascular response in many amphibians far exceeds their total histamine content Any general physiological significance of histamine in relation to the control of amphibian vascular smooth muscle is therefore rather unlikely but despite this small local vascular beds may of course respond to histamine

The main tissue histamine stores of amphibians like those of most fish (Reite 9a b) are apparently located in the stomach although a few amphibian species ive been found in which histamine occurs in large quantities in the skin together with 5 hydroxytryptamine and other amines (Erspamer Roseghini and Cei 1964 Erspamer *et al* 1966 Observations have not yet been made on the vascular response of the latter species to histamine

With respect to amphibian extravascular smooth muscles the effect of histamine have been studied in preparations from the lung (Carlson and Luckhardt 1920—21 Luckhardt and Carlson 1920—21 Verzar 1940 Kadatz 1949 50 Kobayasi and Furuya 1960 from the alimentary tract (Lio 1927 Feldberg and Schilf 1930) from the urinary bladder (Adler 1918) and from the iris (Gautier 1927 Hadjimichalis 1931) Among these preparations taken from different species the lung of the Japanese toad was shown to contract in response to relatively low concentrations of histamine and relax in response to high concentrations (Kobayasi and Furuya 1960) while preparations from all other species showed either no response to histamine or only weak contraction or relaxation It seems justifiable to conclude that both vascular and extravascular smooth muscles of amphibians generally show a low sensitivity to histamine.

The blood pressure decrease following intravascular administration of 5 hydroxy

tryptamine was not accompanied by any noticeable changes in heart rate (Fig 1) Furthermore the depressor effect of 5 hydroxytryptamine remained after complete blockade of the responses to catecholamines and acetylcholine whereas it was abolished by methysergide These experiments show that 5 hydroxytryptamine brings about vasodilatation in most amphibian species and indicate that the vasodilatation is produced by a direct inhibitory action on vascular smooth muscle Vasoconstriction in response to 5 hydroxytryptamine was most marked in some specimens of common toad but the results obtained in perfused blood vessels suggest that this is a common response in many species although the sensitivity may be very low Since effective blockade of the constrictor response was not achieved with any of the tested pharmacological blocking agents its nature remains uncertain

Compound 48/80 and polymyxin B which as far as we know owe most of their vascular actions in mammals to the ability of histamine release through mast cell depletion (Paton 1951, Riley 1959) may apparentl also release 5 hydroxytryptamine from amphibian skin However there is no evidence that amphibians store 5 hydroxytryptamine or other vasoactive agents in their mast cells The present study confirms previous reports (Erspamer 1954 Welsh and Zipf 1966) which state that the 5 hydroxytryptamine in amphibian skin is associated with the cutaneous glands Erspamer (1954 1961) considers the 5 hydroxytryptamine in the skin of amphibians to be designed exclusively for external secretion The presently obtained evidence for release of 5 hydroxytryptamine not only towards the surface of the skin but also into the circulatory system perhaps via the external surface points to a possibility of other functions (particularly in the control of cutaneous gas exchange through influence on the blood vessels) It should be noted however that with respect to effects of compound 48/80 and polymyxin B it is very difficult to evaluate the role played by released endogenous 5 hydroxytryptamine as opposed to the roles played by other indirect or direct actions This also applies to colour changes stimulation of cutaneous secretion and effects on the general condition of the animals Colour changes in frogs in response to 48/80 and polymyxin B have recently also been observed by others (Bhide and Gupta 1967) Since marked actions of compound 48/80 and polymyxin B are present even in the American bullfrog and the mudpuppy which have low levels of 5 hydroxytryptamine in their skin (Welsh 1964 Welsh and Zipf 1966) there is in fact good reason to believe that many of the actions of these agents may be brought about independently of released 5 hydroxytryptam ne The present work ndicates that one of the ways in which they act is mediated through stimulation of α-adrenergic receptor mechanisms

A comparison of the vascular actions of 5 hydroxytryptamine in amphibians with those of histamine in mammals shows many similarities It seems likely that Grant and Jones (1927—29) who were searching for a tissue vasodilatator substance (H substance cf Lewis 1927 and Krogh 1929) and succeeded in preparing an active extract from frog tissues were in fact dealing with 5 hydroxytryptamine However in addition to 5 hydroxytryptamine the tissues of amphibians especially the skin contain several other biologically active indolealkylamines (Erspamer 1961) and also

bradykinin like polypeptides (Erspamer Bertaccini and Cei 1962 Anastasi Erspamer and Bertaccini 1965), and vasodilatator responses to bradykinin were presently found in both species of frog where this agent was tested

There are few previous reports on the pharmacological actions of 5 hydroxytryptamine on amphibian smooth muscle preparations The review of Erspamer (1954) cites some studies in perfused hind legs of frogs and toads which show no effect from 5 hydroxytryptamine or slight vasoconstriction after high doses and these observations are also in agreement with another study in the frog (Kuriaki and Inoue 1955) In extravascular smooth muscles from frogs 5 hydroxytryptamine is shown to induce marked relaxation of the lung (Brecht and Jeschke 1962) and rhythmic movements and increase in tone in rectum and intestine (Vogt 1954 Fange 1962) The present results do not disagree with those published previously but indicate that occasionally the vasoconstrictor effect of 5 hydroxytryptamine may be quite strong at least in some species The failure of review articles (Erspamer 1954 1961 1966) to call attention to the powerful inhibitory actions of 5 hydroxytryptamine on vascular smooth muscle which are demonstrated in this work has simply been due to lack of experimental data

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Hemodynamics in the Bone Marrow of Anemic Rabbits with Increased Hematopoiesis

By

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Abstract

MICHELSEN, K, *Hemodynamics in the bone marrow of anemic rabbits with increased hematopoiesis* Acta physiol scand 1969 77 52—57

In fifteen rabbits hematopoiesis was stimulated by a grave phenylhydrazine anemia When the hematopoietic activity was maximal as judged from the reticulocyte counts, 6 or 7 days after the first injection of phenylhydrazine, the left femur was perfused in situ through the nutrient artery by blood led through an extracorporal perfusion arrangement from the left carotid artery of the anesthetized animal The blood flow, the nutrient artery perfusion pressure and the intramedullary venous pressure were recorded The bone marrow blood flow was greater and varied less than in non anemic animals The arterial vessels exhibited a weak ability to autoregulate flow and acetylcholine caused arterial dilatation showing that neither the grave anemia nor the great hematopoietic activity had brought about a maximal dilatation of the arterial vessels *The venous resistance to bone marrow blood flow was constantly small whereas it has previously been shown to vary greatly in non anemic animals*

Most investigators who have attempted to determine quantitatively the blood flow through bone marrow (Drinker Drinker and Lund 1922 Cumming 1962 Cumming and Nutt 1962 Held and Thron 1962 Breuer, Hirsch and Sachweh 1964, Breuer and Hirsch 1964) have observed great differences in flow from one experiment to another Cumming (1962) suggested that these differences were due to differences in content of red bone marrow tissue In previous works on the hemodynamics of the bone marrow circulation (Michelsen 1967, 1968) it was found that not only the blood flow, but also the intramedullary venous pressure and the relationship between pressure and flow varied considerably from one preparation to another

In the present work the blood flow through the nutrient artery of the rabbit femur the perfusion pressure in the nutrient artery and the intramedullary venous pressure of the femur have been determined in animals in which an acute anemia had been induced by injection of phenylhydrazine This made possible an analysis of the basic hemodynamics of bone marrows in similar states of hematopoietic activity and at the same time bone marrow hemodynamics in an acute grave anemia could be studied

Methods

The experiments were carried out on 15 mature rabbits of both sexes weighing between 3 0

erythrocytes were counted in an electronic particle counter (Celloscope Model 101 A B Ljungberg & Co, Stockholm, Sweden) The hematocrit ratio was determined by means of a microcapillary centrifuge (Model MB International Equipment Company, Boston, Mass, U S A.) The reticulocytes were counted in smears vitally stained by brilliant-cresyl-blue and after stained by Giemsa

knee joints

The systemic arterial pressure was measured through an indwelling catheter in the right femoral artery This and oth

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was prevented by intravenou

was avoided by removal of

1968) The nutrient artery and vein of the left femur were dissected out and cannulated A thin nerve accompanying these vessels, was cut The catheter in the nutrient vein was connected to a pressure transducer for measurement of intramedullary venous pressure The

went through a Starling resistance to the right jugular vein of the rabbit The perfusion

was used The acetylcholine concentration was 1 mg/ml and the infusion rates varied between 0 01 and 0 03 ml/min

At the end of each experiment the femoral bone was cleansed of soft tissue and carefully crushed The marrow tissue was taken out weighed and examined macroscopically

Results

Before the injections of phenylhydrazine, the different animals had hemoglobin concentrations between 12 1 and 15 8 g per 100 ml venous blood, hematocrit ratios between 0 37 and 0 43 and erythrocyte counts between 4 8 and 6 1 millions per mm^3

On the day of operation the hemoglobin concentrations were between 6 1 and 9 2 g per 100 ml venous blood, the hematocrit ratios between 0 18 and 0 27 and the erythrocyte counts between 1 9 and 3 5 millions per mm^3 These figures show that the animals were profoundly anemic The range in reticulocyte counts had changed from between 0 6 and 4 0 per cent to between 19 and 30 per cent, indicating that a high degree of hematopoietic activity was present



Fig 1

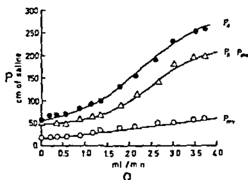


Fig 2

Fig 1 Blood flow through the nutrient artery of in situ perfused rabbit femurs at perfusion pressures in the nutrient artery between 110 and 150 cm of saline Flow values given in ml/min × g wet marrow tissue The symbol ▲ represents values found in the present series of anemic animals The symbol ○ represents values found in a previous series of experiments on non anemic rabbits (Michelsen 1968)

Fig 2 Pressure—flow relationships from a rabbit femur preparation perfused through the nutrient artery Flow (Q) has been plotted versus perfusion pressure in the nutrient artery (P_a), versus intramedullary venous pressure (P_{mv}) and versus the pressure difference between perfusion pressure and intramedullary venous pressure (P_a P_{mv})
The perfusion pressure has been corrected for the pressure fall in the nutrient artery cannula

The weight of the femoral bone marrow from the individual anemic animals was between 1 3 to 2 1 g wet weight As judged from the macroscopic examination, the femoral bone marrow of the individual animals were in similar, marked states of activity The bone marrow tissue was softer and more deeply red than in non-anemic animals Regions with yellow marrow were present in the distal metaphyses The yellow marrow regions were more sharply delineated than in non-anemic rabbits

The blood flow through the nutrient artery at perfusion pressures close to the systemic blood pressure of the respective animals of the present series are given in Fig 1 in ml per min and g wet marrow tissue Fig 1 also includes the flow values found in a previous series of similar experiments performed on non-anemic animals (Michelsen 1968) It should be noted that when the catheter in the nutrient vein was opened to atmosphere the blood flow through the nutrient artery increased only insignificantly This shows that any possible element of venous stasis brought about by the occlusion of the outflow through the nutrient vein was ignorable

In the present series of anemic animals the mean blood flow through the nutrient artery at perfusion pressures near the systemic arterial pressure (Fig 1) was 1 21 ml per g wet marrow tissue per min (S D 0 18) as compared to 0 47 ml per g wet marrow tissue per min (S D 0 27) in non anemic animals This difference is significant on the 0 01 per cent level Furthermore the individual differences were less in the anemic than in the non-anemic group (Michelsen 1968) The variances of the results in the two groups (compared by use of the formula $F = S_1^2/S_2^2$ where S_1^2 is the variance of the flow values in the non anemic group, and S_2^2 the variance of the flow values in the anemic group) were significantly different at the 5 per cent level

In 12 out of 15 expts a small retrograde blood flow was observed from the nutrient artery catheter when this was opened to atmosphere In non anemic animals a retrograde blood flow was observed in only 7 of 44 expts

The intramedullary venous pressure at perfusion pressures between 110 and 150 cm of saline was constantly below 40 cm of saline in the present series of experiments whereas it varied between 10 and 80 cm of saline in the non anemic rabbits

Changes in the bone marrow flow and perfusion pressure were accompanied by changes in the intramedullary venous pressure However these changes were small whereas in the non anemic group the changes in venous pressure following changes in flow and perfusion pressure could be great

Whereas the relationships between different steady state values of perfusion pressure (P_a) intramedullary venous pressure (P_{mv}) and blood flow (Q) varied greatly from one experiment to another in the series of non anemic rabbits (Michelsen 1968) these relationships were uniform and of the type illustrated in Fig 2 in all experiments of the present series Fig 2 shows that the curve relating perfusion pressure (P_a) to flow is slightly convex against the flow axis in the pressure range between 50 and 130 cm of saline Above 130 cm it is convex against the pressure axis The intramedullary venous pressure (P_{mv}) increased moderately with increasing flow and perfusion pressure and the curve for (P_a—P_{mv}) versus flow has the same shape as that for perfusion pressure versus flow

Addition of acetylcholine (at rates between 1 6 and 4 8 μg/10 sec) to the arterial blood entering the nutrient artery increased the blood flow through the nutrient artery sometimes by 100 per cent and elevated the intramedullary venous pressure by 20—30 cm of saline

Discussion

It is of interest to compare the basic hemodynamics of the bone marrow circulation in animals with great hematopoietic activity to the bone marrow hemodynamics in animals with presumably normal hematopoietic activity The flow values found in the present experiments on anemic animals as well as those found in the experiments on non anemic animals (Michelsen 1968) should not without reservations be considered as estimates of the bone marrow blood flow in intact animals for several reasons The method used for perfusion of the bone marrow may reduce the vascular tone of the bone marrow vessels (Michelsen 1968) Furthermore the cutting of the nerve accompanying the nutrient vessels to the femur (see methods) will probably cause a dilatation of the arterial vessels since the bone marrow is suppl ed by vaso constrictor fibres from the sympathetic nervous system (Weiss and Root 1959) Finally the femoral bone marrow of rabbits though supplied predominantly by the nutrient artery is supplied also by arteries which enter the bone marrow in the metaphyseal regions (de Marneffe 1951 Brookes and Harrison 1957)

The present finding of a greater blood flow in the anemic than in the non anemic animals is in accordance with the finding of Linke *et al* (1965) of a greater out

flow through the nutrient vein of femurs in rabbits made anemic by bleeding than in non anemic rabbits The small individual differences in flow in the group of anemic animals as compared to the group of non anemic animals (Fig 1) were probably due to the fact that in the anemic animals the bone marrows were in more similar states of hematopoietic activity In the non anemic animals the hematopoietic activity differed considerably from one animal to another, as judged from the macro scopical examination of the bone marrows

Since a retrograde blood flow through the nutrient artery was present more frequently in the anemic than in the non anemic animals, functional arterial anastomoses between the ramifications of the nutrient artery and arteries that enter the bone marrow in the metaphyseal regions (de Marneffe 1951, Brookes and Harrison 1957) were present more often in the anemic animals

The bone marrow circulation of the anemic animals also differed from that of the non anemic in that the intramedullary venous pressure, as measured through the nutrient vein of the femur, was constantly low The combination of a large blood flow and a low intramedullary venous pressure implies that the venous resistance to bone marrow blood flow was small so that flow was predominantly determined by the resistance in the arterial vessels

The convexity of the curve relating (P_a—P_{mv}) to flow (Fig 2) between pressures of 50 and 130 cm of saline illustrates that a weak autoregulation was present in this pressure range in the anemic animals and the increase in flow and intramedullary venous pressure caused by acetylcholine shows that this substance caused a dilatation of the arterial vessels Similar findings were made in the non anemic animals (Michelsen 1968)

The bone marrow blood flow (Q) is determined by the equation (I) $Q = \Delta P/(R_a + R_v)$ (where ΔP is the pressure fall in the bone marrow vascular bed R_a the arterial resistance and R_v the venous resistance) The difference in flow between the anemic and non anemic group of animals can therefore partly be explained hemodynamically by the low venous resistance in the anemic animals In accordance with this the bone marrow blood flow in non anemic animals was usually great when the venous resistance was small (Michelsen 1968)

The venous resistance (R_v) was less than the arterial resistance (R_a) in all but 4 of the 44 non anemic animals at perfusion pressures between 110 and 150 cm of saline (Michelsen unpublished data) This means according to equation (I) that the low venous resistance found in the anemic group could not account for the 2 5 times greater bone marrow blood flow in these animals Also the arterial resistance must therefore have been smaller

The present experiments cannot identify the factors which are responsible for the decrease in arterial and venous resistances observed when the bone marrow is stimulated to increased hematopoiesis by an acute anemia Apart from the possible significance of rheological differences between anemic and non anemic blood metabolic changes and changes in tissue and vascular architecture are probably important Thus both the low oxygen capacity of the anemic blood and the increased metabolic

rate, which was probably present due to the increased hematopoietic activity, may have caused the decrease in arterial resistance Whether the decrease results from dilatation of the resistance vessels, formation of new vessels or both, cannot be decided However, the fact that acetylcholine decreased the arterial resistance indicates that neither the grave anemia nor the great hematopoietic activity had brought about a maximal dilatation of the arterial vessels

The low venous resistance found cannot be due to changes in the vascular tone of the veins, since the bone marrow venous system lacks smooth muscle (de Marneffe 1951) It is suggested that the decrease in the venous resistance was due to an increase in the dimensions of the draining part of the bone marrow circulatory system Such an increase could result from a decrease in the amount of parenchymatous tissue, due to an acute release of cells from the bone marrow to the blood Transformation of yellow bone marrow to red bone marrow might also lead to a decrease in the venous resistance since the vascularity of the red bone marrow tissue is much greater than that of the yellow (Brånemark 1959)

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Conditional Reflex Activation of the Sympathetic Cholinergic Vasodilator Nerves in the Dog

By

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Abstract

BOLME, P and J NOVOTNY *Conditional reflex activation of the sympathetic cholinergic vasodilator nerves in the dog* Acta physiol scand 1969 77 58—67

Sympathetic, cholinergic vasodilatation in skeletal muscle was induced as orienting and condi-

[illegible]

dilator system is activated prior to situations when an increased muscle blood flow is suddenly required The results also suggest that activation of the dilator nerves takes place not only in emotionally charged situations, such as the defence reaction, but also in anticipation of exercise when the emotional involvement is less prominent

In conscious dogs electrical stimulation of the sympathetic vasodilator areas in the brain elicited an increased muscle blood flow, a tachycardia and a slight rise in the arterial pressure (Bolme *et al* 1967) In addition such stimulation frequently increased alertness to various degrees However, in some experiments stimulation induced circulatory changes without apparent behavioural responses This finding suggested that the sympathetic vasodilator mechanism might be activated during different physiological situations of both emotional and non-emotional origin This concept seemed to differ from the opinion of Abrahams Hilton and Zbrożyna (1960) who claim that in cats the vasodilator areas in the diencephalon and the mesencephalon are reflex centres for emotionally tinged defence reactions However, Abrahams Hilton and Zbrozyna (1964) obtained by electrical stimulation in the brain stem in conscious [illegible] sympathetic vasodilatation together with mild behavioural

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changes They concluded that sympathetic vasodilatation might be part of preparatory alerting reactions, which is in agreement with our own hypothesis (Bolme *et al* 1967)

From experiments in anesthetized cats Eliasson *et al* (1951) suggested that the sympathetic cholinergic vasodilator nerves are activated preparatory to any reaction requiring increased muscle blood flow To test the validity of this hypothesis, a series of experiments were performed on conscious dogs, in which sympathetic cholinergic vasodilator responses were evoked as conditional reflexes to stimuli with and without emotional backgrounds

Abrahams, Hilton and Zbrozyna (1964) showed that a vasodilatation in the muscle of the cat could be elicited as a conditional response to electrical skin stimulation This vasodilatation was thought to be due to activation of sympathetic cholinergic vasodilator nerves We also used electrical skin stimulation to evoke a response of emotional origin similar to the defence reaction or 'die affektive Abwehr reaktion' as originally described by Hess and Brugger (1943) To elicit a reaction with a minimum of emotional background we used a conditional reflex based on a sound stimulus followed by exercise on a treadmill

Methods

The experiments were carried out on 17 mongrel dogs weighing 8—21 kg Each experiment was scheduled individually but in principle the study was performed in the following way

1 *The screening period* usually lasting no longer than 14 days During this time dogs were trained to stand in the sound proof box in which all experiments were performed. In this way we could select those dogs which learned to stand quietly in the box without getting excited during the test periods usually 1/2—2 hrs The dogs in the moderately illuminated box were continuously watched from the outside through a one way mirror

2 *The initial training period* During this time the dogs which had passed the screening test were trained in their final program The conditional reflexes were built up during this period lasting from 2 weeks to 4 months The training was considered complete when the conditional and the un-conditional stimuli gave rise to adequate alertness responses characterized by raising the head and looking around with a general attitude of expectation

3 *The surgical operation* When the initial training period was finished the dogs were provided with equipment for the measurement of hind limb blood flow arterial pressure and heart rate by an aseptic operation

4 *The experiment period proper* After a post-operative recovery period of 2—10 days the cardio-vascular responses to the conditional and the un-conditional stimuli were recorded No experiment was performed if the dog showed any sign of pain or impaired motor activity whilst running

The conditional reflex training program

The conditional stimuli signals were produced from a tone generator and amplified by loud speakers in the box The frequencies of these sounds could be varied The unconditional stimuli were of two types The first consisted of an electrical skin stimulation produced by passing an electrical current through two needles placed subcutaneously close to each other The stimulation areas were either on the back of the dog or in a front leg The electrical currents were supplied by a Grass S 6 stimulator and consisted of square wave pulses with a frequency of 5—10 imp/sec a duration of 1—10 msec and an intensity of 5—20 V In the training and during the experiments the tone was sounded for 10—15 sec The electrical skin stimulation was started 3—10 sec after starting the tone and both stimuli were turned off simultaneously In some experiments tones with different frequencies were combined h

electrical skin stimuli of varying intensities The other type of un conditional stimulus was

tional stimulus alone was tested intermittently during the whole training and experimental periods and the conditional stimuli were given to the dogs randomly in relation to the combined program of conditional/unconditional stimuli

The surgical operation and the measuring devices

After the initial training period had been completed the dogs were anesthetized with pento barbital (30 mg/kg i v) By means of a laparatomy the tip of a polyethylene catheter (Clay Adams PE 205) was inserted into the aorta for the future measurement of arterial pressure An electromagnetic flowmeter probe was fitted on one of the external iliac arteries The free end of the polyethylene catheter and the leads from the probe were passed subcutaneously to the interscapular region emerging through the skin where they were fixed To enable occlu sion of the iliac artery for the estimation of zero blood flow a silk thread was placed around the artery and the endings were passed into the groin where they were fixed to the skin

After the post operative recovery period the experiments were performed in the sound proof box To measure the arterial pressure the implanted aortic catheter was connected to a Statham transducer (P 23 AC) To determine the hind limb blo

probe were connected to an electromagnetic flowmeter

sine wave excitation The heart rate was recorded by

pressure or the flow signal (Goldschmidt and Lindgren

Grass model 5 polygraph Vascular resistance in the hind leg was calculated from the mean blood pressure (MBP) and the blood flow (BF)

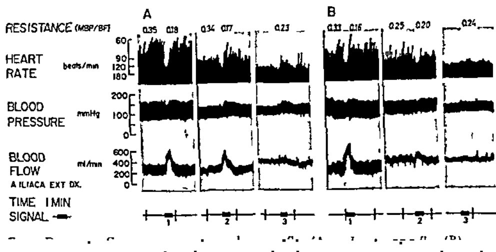
Drugs were usually administered through the aortic catheter Atropine (0 03—0 2 mg/kg) was given to test that cholinergic mechanisms had been involved In some experiments propra nolol (0 6—1 4 mg/kg) was given either before or after the atropine injection to see if β receptor blockade had any influence on the response

Results

Orienting reflexes

The influence of the sound to be used in the study as the conditional stimulus on the circulation and behaviour was tested in four dogs These dogs were operated directly after the screening period When the sound was introduced to them they were all alerted The circulatory effects were a slight increase in arterial pressure an increase in the hind limb blood flow and a tachycardia The increase in blood flow could be completely or partially blocked by atropine (0 05—0 1 mg/kg) slowly injected into the aortic catheter In two dogs a slight vasodilatation remained in response to the sound stimulus This effect could be abolished by propranolol (1 2—1 4 mg/kg) in fused into the aortic catheter The blocking effect was observed after doses that in duced a bradycardia and an abolition of the sound induced heart effect Smaller doses of propranolol (0 6—1 2 mg/kg i a) did not block the vasodilatation caused by the sound whether the drug was added before or after atropine This dose is how ever known to be sufficient for a local β receptor blockade in the hind limb muscles of the dog (Shanks 1967)

Our results indicate that the vasodilatation following the sound was caused by ac tivation of sympathetic cholinergic vasodilator nerves and not by activation of β receptors in the hind limb After 2—4 days of daily exposure to the sound alone the dogs did not respond t this stimulus i e they had become habituated to the sound (Fig 1B)



1 Sound stimuli "day 1" (the first day that the animal was exposed to the orienting stimulus)
2 Sound stimuli "day 4"
3 Sound stimuli "day 4", after atropine 0 03 mg/kg i a
Note that the dog became habituated to the orienting but not to the conditional stimulus

Conditional reflexes

a *Electrical skin stimulation* Five dogs were conditioned to electrical skin stimulation The experiments were performed during a period of 7—30 days after the operation The response to the conditional stimulus alone was tested at least once every day The conditional reflex response, *i e* the effect on the signal alone, was similar to the initial orienting reflex described above, in respect of both the behavioural and the circulatory events However, habituation was never seen to the conditional stimulus

A representative experiment is seen in Fig 1 Section A shows the response to the conditional stimulus alone (250 cps) on the 4th and the 7th day of post-operative training The effects were more or less identical on the two occasions an increased alertness, a tachycardia a slight rise of the arterial pressure, a doubling of the iliac blood flow, and a 50 per cent decrease in the peripheral resistance The effect on the blood flow was abolished by atropine (0 03 mg/kg i a.) Section B shows an orienting reflex response The dog responded initially to this new sound (500 cps) in the same way as to the conditional stimulus (*cf* A 1 and B 1) After 3 days however the dog had become habituated to the 'non conditioning' sound This experiment revealed that the dogs were able to discriminate between two sounds of different frequencies

This finding led us to design experiments in two dogs, in which sounds of different frequencies were followed by electrical skin stimulations of varying intensities One such experiment is shown in Fig 2 The response to the 250 cps signal (A1), associated with the weaker electrical skin stimulation was an increased alertness, a slight

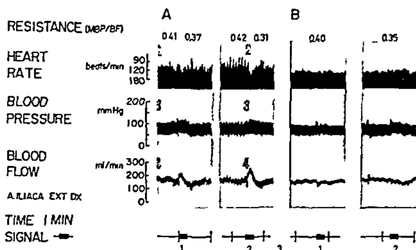


Fig 2 Dog 11 kg Conditional reflexes to electrical cutaneous stimulation Differentiation
1 Sound 250 cps (conditioned to 7 V 20 imp/sec 10 msec)
2 Sound 600 cps (conditioned to 15 V 40 imp/sec 10 msec)
3 Atropine 0 02 mg/kg i.a
Note the graded blood flow responses, completely abolished by atropine

tachycardia, a blood pressure rise and an increase in the blood flow, from 165 to 205 ml/min The peripheral resistance decreased from 0 41 to 0 37 The 600 cps signal (A 2), associated with the stronger skin stimulation gave rise to an increase in alertness not noticeably different from that seen with 250 cps The tachycardia and the blood pressure rise were similar to the effects seen after the 250 cps signal However, the blood flow increase was more marked in response to the 600 cps signal, from 160 to 250 ml/min and the peripheral resistance decreased from 0 42 to 0 31 Thus, the conditional reflex response could be varied with the intensity of the un conditional stimulus The conditional reflex vasodilatation could in this way be graded The blood flow increases following the two different signals were abolished by atropine (0 02 mg/kg i a)

b *Exercise* Nine dogs were conditioned to exercise on the treadmill The initial training period before the operation, lasted 1—4 months Five dogs were trained at one speed only chosen so as to make each dog run almost as fast as it could The other four dogs were trained at two different speeds After the operation the training was started again after a recovery period of 4—14 days One of the experiments is shown in Fig 3 At A the conditional stimulus followed by the exercise (1) evoked an increase in the arterial pressure from 170/100 to 180/115 and a moderate tachycardia The blood flow increased from 115 to 210 ml/min and the peripheral resistance decreased from 1 1 to 0 65 After the conditional stimulus alone (2) the dog was alerted some tachycardia appeared and the arterial pressure increased from [illegible]/90 to 170/105 The blood flow increased from 105 to 180 ml/min and the peripheral resistance decreased from 1 1 to 0 71 Thus the conditional reflex blood flow was of nearly the same magnitude as that caused by the exercise

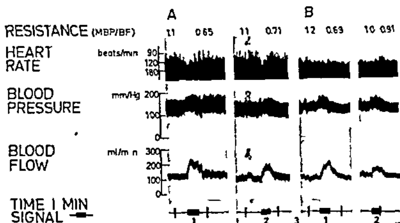


Fig 3
1
2
3

After atropine (0 04 mg/kg i a), the response to the sound and treadmill together (B 1) was a rise in arterial pressure from 150/110 to 170/120 and no change in heart frequency, presumably due to the tachycardia already produced by the atropine The blood flow increased from 100 to 200 ml/min and the peripheral resistance decreased from 1 2 to 0 69 Thus the response to the exercise was not quantitatively changed but the blood flow rise was less abrupt after atropine It did not begin when the sound commenced but when the treadmill started and was gradually built up during the exercise period

The conditional reflex response (B 2) was changed after atropine The blood flow increased only 25 per cent and the reduction in the peripheral resistance value was 9 per cent No tachycardia was seen but the arterial pressure increased slightly more than before atropine This suggests that the main part of the vasodilatation seen before atropine in response to the conditional stimulus was caused by activation of sympathetic cholinergic vasodilator nerves

In order to confirm that the vasodilatation was not merely a part of a general alerting reaction differentiation experiments were made Four dogs were trained to run at two different speeds following two different signals The speeds were chosen so that one was almost maximal for the dog and the other moderate The differentiation program was started at least two weeks before the operation

In Fig 4 one such experiment is shown In A the responses to the two conditional stimuli before atropine can be seen In response to 2500 cps (1) associated with the moderate exercise a small arterial pressure rise and a blood flow increase from 95 to 205 ml/min was observed The peripheral resistance decreased from 1 5 to 0 71 The sound 250 cps (2) associated with the heavy exercise was followed by a small rise in arterial pressure and a blood flow increase from 95 to 240 ml/min The pe

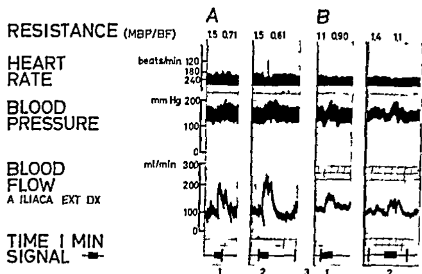


Fig 4 Dog 10 kg Conditional reflexes to exercise on treadmill Differentiation
1 Sound (2500 cps) conditioned to 30 m/min
2 Sound (250 cps) conditioned to 80 m/min
3 Atropine 0 2 mg/kg i a

ripheral resistance value decreased from 1 5 to 0 61 Thus the conditional vasodilatation was most pronounced in response to the 250 cps signal, conditioned to the heavier exercise In this experiment the effects on the heart rate caused by the signals were small or absent probably due to the very high resting heart frequency After atropine (3) the vasodilatations following the conditional stimuli were very much reduced In respone to the 2500 cps signal only a 25 per cent blood flow increase was seen and the peripheral resistance decreased less than 20 per cent The 250 cps signal led to a 45 per cent blood flow increase and the peripheral resistance decreased about 20 per cent The effects on the arterial pressure following the signals were not diminished by atropine If anything the blood pressure rises were slightly greater after atropine than before probably as a result of the reduced vasodilatation in skeletal muscle together with a vasoconstriction in *e g* cutaneous and splanchnic vessels

Discussion

Studying cardiac conditional reflexes in the dog Gantt (1960) reported that the heart rate was very labile and was changed more by emotional stimuli than by motor activity We found that also the resting hind limb blood flow changed considerably under the influence of rather small environmental changes *e g* a person entering the room or the dropping of a pencil on to the floor In the same way, Abrahams Hilton and Zbrozyna (1964) observed in cats that muscle vasodilatation was easily elicited by natural stimuli This fact made it necessary for us to perform this study

with the dogs in a completely sound proof conditioning chamber in order to get as uniform and comparable responses as possible However, despite this precaution the basal conditions in the same dog changed from time to time even during the same experimental period and the response to an identical stimulus therefore differed The reasons for these differences in reactivity were never obvious As a consequence of this, in the differentiation experiments we have tried to make "paired observations" *i e* compared the responses to the different conditional stimuli, given in a random order and within a short time interval when the basal conditions were considered to be as near identical as possible

Activation of the sympathetic cholinergic vasodilator nerves appeared to be a part in the orienting reflex following an unspecific sound stimulus The hind limb blood flow could increase at least two-fold in response to the sound Most of this vasodilatation was abolished by atropine i a Occasionally there persisted, even after atropine, a blood flow increase which could further be reduced by propranolol i.a if given in doses high enough to recirculate and block the heart responses However, smaller doses of propranolol, although sufficient for β receptor blockade in the hind leg, did not influence the blood flow increase This suggests that the hind limb blood flow increase in the orienting reflex, and probably also in the conditional reflex, was due primarily to the activation of sympathetic cholinergic vasodilator nerves and that an increased cardiac output, not measured in our study, also contributed to the effect An increased cardiac output might well have been caused by activation of the same CNS structures responsible for the sympathetic cholinergic vasodilatation in the hind limb Rosen (1961) showed that the contractile force of the heart increased following stimulation of the hypothalamic vasodilator nervous outflow Moreover, Andersson and Brown (1967) and Folkow *et al* (1968) have reported an increased cardiac output in dog and cat following stimulation of the sympathetic vasodilator outflow in the hypothalamus

In the present study the blood flow through the external iliac artery was measured The sympathetic cholinergic vasodilator nerves are considered to innervate only the vessels of the skeletal muscles (Uvnas 1960), and the external iliac artery supplies blood to areas other than muscular tissue Bolme *et al* (1967) reported no difference in the type of blood flow response when the vasodilator nerves were activated in the hypothalamus between experiments with intact or skinned hind limbs However quantitative differences were observed indicating that if the blood flow rise in the intact hind limb was 60—70 per cent, the true muscle blood flow increase was 100—150 per cent This would mean that in our study the blood flow increases to the hind limb muscles were under estimated since they were measured as iliac blood flow rises

The conditional reflex blood flow response could be graded with either type of unconditional stimulus used The response to the skin stimulation may support the hypothesis of Abrahams Hilton and Zbrożyna (1960, 1964) that the sympathetic cholinergic nerve fibres are activated to produce cardiovascular adjustments in the defence reaction The experiments with graded conditional reflex responses to dif-

ferent speeds on the treadmill show that the sympathetic vasodilator nerves are also activated to produce vasodilatation in situations where the emotional involvement is not prominent The blood flow rise preparatory to exercise can hardly be considered as just a part of an emotional alerting reaction The fact that the vasodilatation was more intense in response to the sound indicating vigorous exercise than to that indicating moderate work lead us to believe that the vasodilator nerves were activated to prepare the animal for the exercise Recently, Hilton (1968) has expressed a similar opinion claiming that the cardiovascular events preparatory to exercise, including activation of the vasodilator nerves in skeletal muscles, can be elicited independently of the defence reaction

The sympathetic vasodilatation is presumably of importance just for short periods of time preparatory to exercise After the work has started metabolic vasodilator factors probably take over the regulation of the muscle blood supply One indication that activation of the sympathetic vasodilator nerves is not of importance during exercise is the finding that the blood flow increase to the skeletal muscles during exercise was not reduced when the vasodilator nervous response was blocked by atropine Furthermore, Hirvonen and Sonnenschein (1962) found in the cat the contractile force of the muscle in response to motor nerve stimulation to be the same whether the sympathetic vasodilator nerves were concomitantly activated or not

The question still remains whether or not sympathetic vasodilator nerves are required to produce an adequate increase of skeletal muscle blood flow prior to work or in response to emotional stimuli Smith and Stebbins (1965) could, as a conditional reflex to an electrical cutaneous shock, increase the heart rate and the blood flow in the abdominal aorta of monkeys indicating that vasodilatation took place in the hind limb skeletal muscles They did not study the influence of atropine on this response but according to yet unpublished data (Bolme, Novotny, Uvnas and Wright, to be published) the skeletal muscle vessels of the monkey are not innervated by sympathetic cholinergic vasodilator nerves For this reason the vasodilatation observed by Smith and Stebbins (1965) was probably caused by mechanisms other than the sympathetic vasodilator nerves *e g* an increased arterial pressure and a release of adrenaline from the adrenals

The present study has thus shown that in the dog cholinergic sympathetic vasodilator nerves of the hind limb muscles are activated as a conditional reflex to exercise and cutaneous electrical stimulation This conditional reflex vasodilatation may apparently occur in a variety of situations as a response to stimuli of different types

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The Firing Pattern of Dorsal Spinocerebellar Tract Neurones during Inhibition

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Abstract

JANSEN, J K S, K NICOLAYSEN and L WALLOE *The firing pattern of dorsal spinocerebellar tract neurones during inhibition* Acta physiol scand 1969 *77* 68—84

Dorsal spinocerebellar tract neurones adequately activated by Ia afferents from the tibialis anterior (TA) extensor digitorum longus (FDI) or flexor digitorum longus (FDI) muscles are regularly inhibited by group I afferents from the gastrocnemius soleus (GS) nerve The inhibition is maintained throughout the duration of GS nerve stimulation During inhibition the irregularity of firing may be unchanged or moderately reduced Correspondingly the serial dependency between neighbouring intervals may be unchanged or reduced The inhibitory *input usually caused a constant reduction in firing frequency at various levels of excitatory* drive or occasionally a greater reduction at higher levels of excitatory drive The latter effect was associated with increased regularity of firing and reduced serial dependency Comparable inhibitory effects were obtained by repetitive stimulation of the contralateral somato sensory cortex It is suggested that the inhibitory effects can be accounted for by post synaptic inhibitory mechanisms

Certain features of the transfer signals through the Clarke s column relay of the dorsal spinocerebellar tract (DSCT) can be explained in terms of a simple model of the second order neurone (Walloe 1968 Jansen and Walloe 1968) This applies to the discharge pattern of the second order neurones adequately activated through their primary afferent fibres Some of the predictions of the model such as the size of the unitary EPSPs and the degree of convergence of primary afferent fibres on to each second order neurone have been supported by direct record of the synaptic activity of Clarke column cells (Eide *et al* 1969b)

The DSCT neurones also receive inhibitory inputs from primary afferent fibres (Holmqvist Lundberg and Oscarsson 1956 Eccles Oscarsson and Willis 1961 Hongo and Okada 1967 The excitatory coupling of the DSCT relay is known to be highly specific in the sense that a given second order neurone is activated only from particular muscle Some of the inhibitory input is supplied by group I afferent f[illegible] from muscles not directly connected to the second order neurone (Hongo and O[illegible] 1967 Jansen Nicolaysen and Walloe 1967)

Two types of inhibitory mechanisms have been reported to act at the Clarke column synapses The one is the traditional postsynaptic inhibition presumably mediated via one interneurone (Eccles *et al* 1961, Hongo and Okada 1967) The second is a presynaptic inhibition on which the evidence is less direct It consists of reduction in the size of DSCT volleys and excitability changes of the primary afferent terminals following suitable conditioning (Eccles, Schmidt and Willis 1963, Jankowska Jukes and Lund 1965) and of the pharmacological properties of the inhibitory effect (Jankowska, Jukes and Lund 1964)

The purpose of the present experiments was to study the interaction of excitation and inhibition on the firing pattern of DSCT neurones Controlled excitation of these was conveniently obtained by muscle stretch Preliminary studies (Jansen *et al* 1967) suggested that inhibitory effects could regularly be obtained from GS group I afferents acting on signals from stretch receptors of FDL or pretibial flexors Our main experiment has therefore been to study the effect of a constant amount of inhibition (obtained by repetitive, electrical stimulation of group I GS afferents) on TA EDL or FDL second order neurones under varying amount of excitatory drive The results can be interpreted on the basis of the existing model of synaptic excitation of the DSCT second order neurone

Methods

The ... was denervated
... rus (EDL) and
... uld be attached
... nerve was cut
... determined in
... The ipsilateral
ventral roots L VI L VII and S I were always cut The activity of single DSCT axons was
... rded
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... The
duration of the inhibitory stimulus was limited to approximately 10 sec Consequently the number of firing intervals in these periods might be too small to determine the serial correlation coefficient reliably To get a measure of the first order serial dependency we have instead used the linear regression line describing the relationship between the duration of one interval and the average duration of the following interval (see Jansen *et al* 1966)

The slope of this regression line depends on the first order dependency between intervals There is however no unique relation between this slope and the first order serial correlation coefficient but in view of the uniform behaviour of this group of DSCT neurones (Jansen *et al* 1966) it is considered sufficiently accurate for the present purposes

Results

During the experiments to be reported the dorsolateral funicle of the spinal cord was explored by a micropipette to find axons of DSCT neurones monosynaptically activated by group I afferents from TA EDL or FDL and also activated by stretch of the appropriate muscle Once a unit was identified according to criteria previously described (Jansen and Rudjord 1965) the effect of repetitive stimulation of the GS nerve was examined In some preliminary experiments the inhibitory effect of dif-

ferent frequencies of nerve stimulation was determined No certain reduction in firing frequency was found for stimulus rates of less than 10 sec^{-1} At higher rates the inhibitory effect increased approximately linearly up to frequencies of about 120 sec^{-1} Beyond this rate the inhibitory effect was only slightly increased Since the purpose of the present experiments was to study the effect of inhibition on the firing pattern of the neurones, the frequency of stimulation was routinely kept at 100 sec^{-1}, and the shock intensity was maximal for group I afferent fibres in the following experiments If there was noticeable reduction in firing frequency during GS nerve stimulation, the firing frequency of the unit was changed by varying the degree of static stretch of the appropriate muscle and the effect of the same inhibitory stimulus was determined at the various levels of excitatory drive

GS inhibition of TA—EDL Ia in units Most of our material consists of DSCT units activated from primary endings of the TA and EDL muscles Such units are usually inhibited by group I afferent volleys in the GS nerve (Jansen *et al* 1967) In the present series of experiments the effect of group I GS stimulation was examined on 18 TA—EDL Ia units and the firing frequency was perceptibly reduced in 16 of them Seven of these were studied at a reasonable number of different states and for a sufficient period of time to permit a systematic description The data from these seven units constitute most of the material on which the following account is based Supporting data were obtained from the other units

A characteristic example of the effect is presented in Fig 1 Record A shows the unit firing without any load on the tendon This background firing was often quite regular and at a rate of about 10 imp sec^{-1} The cell was silenced immediately by GS nerve stimulation at 100 sec^{-1} and the inhibition persisted throughout the period of stimulation Just after the inhibition there was usually a transient rebound increase in firing frequency With a 50 g load on the TA tendon (Fig 1B) the firing frequency of the unit increased to about 40 imp sec^{-1} The inhibitory stimulus again reduced the firing frequency The reduction was great initially and recovered partly in the following period But the inhibition was clearly maintained throughout the 10 sec of inhibitory stimulation To illustrate the time course of the inhibition the firing frequency of the same unit with a 200 g load on the TA tendon has been plotted in Fig 1C During the inhibitory stimulus the firing frequency dropped immediately and recovered partly during the first few sec Thereafter the inhibitory effect remained approximately constant and the firing frequency of the cell stayed between 50 and 55 imp sec^{-1} till the end of the inhibition

The inhibition illustrated in Fig 1 was due to activation of group I afferent fibres of the GS nerve This was controlled by dorsal root records of the afferent volley The inhibition might be present at shock intensities as low as 120 per cent of group I threshold The inhibitory effect increased with increasing shock strength till all group I fibres were activated Group I maximal shocks were routinely employed in the following experiments

The important observation of Fig 1 from the point of view of firing pattern of the

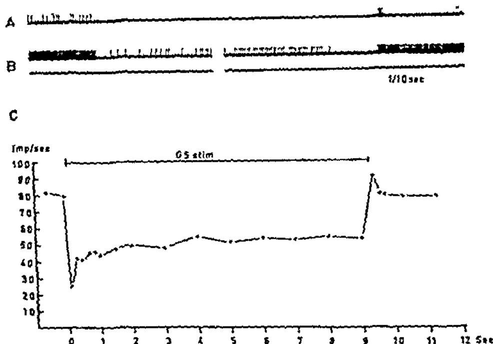


Fig 1 Effect of repetitive (100/sec) GS nerve stimulation on Ia DSCT neuron activated by stretch of TA. Intensity of nerve stimulation just below group I maximum. A No load on TA B 50 g load on TA tendon 7 sec cut out in the middle of record C. Plot of firing frequency against time of same unit during GS inhibition 200 g load on TA tendon

cells is that the inhibition is maintained and gives rise to an approximately steady state firing after the initial overshoot The following statistical description of the firing pattern requires steady state periods to be meaningful Therefore, the first sec of the transient after the onset of inhibition was excluded and interval distribution and serial dependency of the rest of the spike trains was determined The small drift which commonly was present also in the late period of inhibition was ignored, since it will not have any significant influence on the following rather crude statistical description

The distribution of intervals On adequate excitation the DSCT units usually generate unimodal and fairly symmetric interval distributions (Jansen *et al* 1966) During steady state inhibition most units still generated the same type of histograms as long as their mean firing frequency remained above 10—20 imp sec [1] This is illustrated in Fig 2 With a 50 g load on the TA tendon the mean interval was 15 6 msec and the distribution symmetrical (Fig 2A) During GS stimulation with the same load the mean interval increased to 26 2 msec but the distribution retained its general shape (Fig 2B) For comparison the interval distribution obtained with a 20 g load on the tendon is shown in C The mean interval was now 29 9 msec, rather

ferent frequencies of nerve stimulation was determined No certain reduction in firing frequency was found for stimulus rates of less than 10 sec^{-1} At higher rates the inhibitory effect increased approximately linearly up to frequencies of about 120 sec^{-1} Beyond this rate the inhibitory effect was only slightly increased Since the purpose of the present experiments was to study the effect of inhibition on the firing pattern of the neurones, the frequency of stimulation was routinely kept at 100 sec^{-1}, and the shock intensity was maximal for group I afferent fibres in the following experiments If there was noticeable reduction in firing frequency during GS nerve stimulation, the firing frequency of the unit was changed by varying the degree of static stretch of the appropriate muscle and the effect of the same inhibitory stimulus was determined at the various levels of excitatory drive

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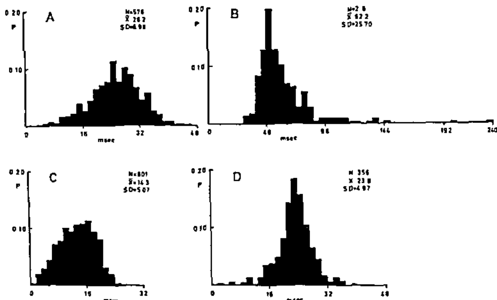


Fig 3 Distribution of intervals during maintained inhibition A Control period 50 g load on TA tendon B as A during 100/sec, group I max GS nerve stimulation C Control period, same unit, 150 g load on TA tendon D, as C during GS nerve stimulation

two situations the unit is firing at approximately the same mean rate, in the one subjected to excitation only (Fig 3A) in the second to a mixed excitation and inhibition (Fig 3D) This type of change was seen consistently for two of the seven TA—EDL units, but as will appear from the following section it is frequently found to a smaller degree in the present material

The irregularity of firing The degree of irregularity of firing is most conveniently measured by the coefficient of variation (CV) which is the ratio between the standard deviation (SD) and the mean interval of the sample Exposed only to its specific excitatory input the coefficent of variation of DSCT cells is usually approximately constant over the entire frequency range (Walloe 1968) This is simply demonstrated by a plot of the SD against the mean interval This plot is well described by a straight line During inhibition the observations would usually obey the same relationship This is illustrated by the data from the unit presented in Fig 4A The data from some of the other units, however, suggested that the firing pattern during inhibition might be slightly less irregular than that of purely excitatory firing For one unit this was definitely so, as illustrated in Fig 4B In this graph the points obtained during inhibition are clearly located below the control values The reduction in variability was accompanied by a change of the interval distribution of the type illustrated in Fig 3C and D

In this type of presentation small systematic changes in CV might be obscured by the random changes in variability from trial to trial The CV of all the inhibitory

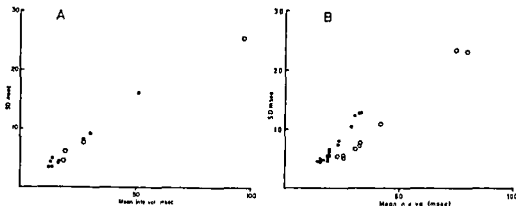


Fig 4 Scatter diagrams of standard deviations against mean intervals of interval distributions during control periods (●) and during GS nerve stimulation (100/sec, group I max) (○) A and B are data from two different units

periods observed in the seven TA—EDL neurones has therefore been plotted against the CV of the immediately proceeding control period of purely excitatory firing (Fig 5) It appears that in all but 5 of 34 inhibitory periods there was a slight to moderate reduction in the CV The data from the unit of Fig 4B is presented with a separate symbol in Fig 5

Accordingly, the conclusion is reached that the irregularity of firing of these DSCT units is usually slightly to moderately reduced by a steady state group I inhibitory input

Serial dependency of intervals When subjected to a purely excitatory input there is a strong negative serial dependency between neighbouring intervals (Jansen *et al*

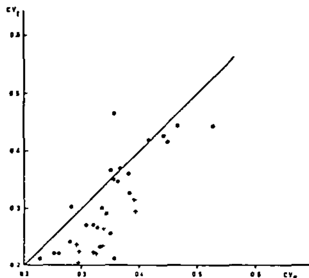


Fig 5 Coefficient of variation

$$\left(CV = \frac{SD}{\bar{x}}\right)$$

during GS inhibition (ordinate) plotted against the CV of the immediately preceding control period (abscissa) Data from seven DSCT Ia units activated by TA stretch Periods with mean frequencies of firing of less than 20 imp/sec excluded + data from same unit as Fig 4B

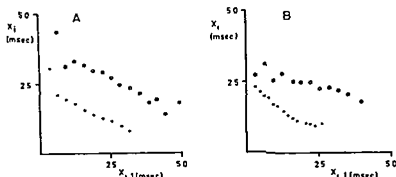


Fig 6 Mean duration of conditional interval distributions (ordinate) plotted against the
… als were 3 2 msec
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… he
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1966) Short and long intervals tend to occur alternately This can be demonstrated by the relationship between the duration of neighbouring intervals, *i e* joint interval histograms In the present work we have determined the average duration of intervals following intervals of a given duration, that is the average value of the conditional distribution of intervals (see Methods) Jansen *et al* (1966) showed that this mean value of the conditional distributions was an approximately linearly decreasing function of the duration of the conditioning interval and that the slope of the regression line was constant at all levels of excitation (their Fig 10) This is due to the fact that the degree of serial dependency is relatively independent of the firing frequency or the excitatory drive of the cell

When inhibition was added to the input the serial dependency was still present For some units the slope of the regression line was unchanged or only slightly reduced indicating that the degree of serial dependency was similar (Fig 6A) as long as the cell was firing at a reasonable frequency (20 to 60 imp sec^{-1} was the range actually observed) For other units the nummerical value of the slope of the regression was definitely reduced (Fig 6B) indicating a decreased serial dependency All our observations on TA—EDL units are presented in Fig 7 which is a scatter diagram of the slope constant of the regression line during inhibition (ordinate) against that of the immediately preceding control period There appears to be different degrees of

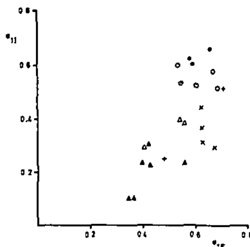
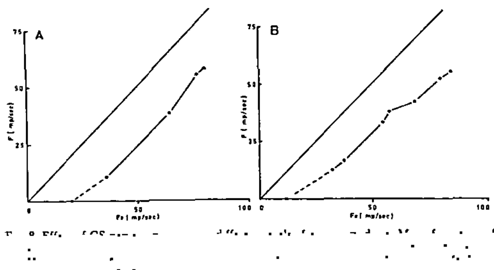


Fig. 7. Slope constants obtained as illustrated in Fig. 6 during inhibition (ordinate) plotted against the corresponding slope constant of the immediately preceding control period (abscissa). Data with mean frequencies of firing less than 30 imp/sec has been excluded. Data from different units have been given separate symbols.

reduction of the slope constant. To show that each unit tended to behave systematically they have been given separate symbols. One group of three units showed no or slight reduction in slope constant (circles). The other units regularly exhibited varying degrees of reduced slope constant during inhibition. Spike trains with mean frequencies of less than 30 imp. sec.[-1] have been excluded from the material.

The main finding of this section is that DSCT units could fire with approximately the same degree of serial dependency during inhibition, but that the serial dependency was frequently reduced. Furthermore, the units with decreased dependency were the ones exhibiting the most pronounced changes in the shape of the interval distributions in the direction of increased regularity of firing. For instance, the data from the unit illustrated in Fig. 3 are represented by x, and those of Fig. 4B by filled triangles in Fig. 7. The unit of Fig. 2 and 4A, on the other hand, is represented by the open circles of Fig. 7.

Inhibition as a function of the excitatory drive. The efficiency of the inhibition was measured as the reduction in average frequency in the steady state period. The results are most conveniently presented as diagrams of the frequency of firing during inhibition against frequency of firing in the control period with the same excitatory drive on the cell (Fig. 8). Since there was always a slow reduction in firing frequency during prolonged excitation the excitatory frequency of firing is given as the mean of the firing frequencies in the period before and after the inhibitory period. This manipulation did not introduce any qualitative changes in the results. If the inhibitory input had no effect, the data in diagrams like those of Fig. 8 would lie on the line through the origin with a slope of unity. This line has been drawn on the plots to illustrate the inhibitory effects. It appears that the observations are reasonably well described by linear regression lines. This applies to all the 7 DSCT units studied. The correlation coefficient was as high as 0.99 for all units. This means



that the observations can be adequately described by a simple equation of the following type

$$F_i = a\ F_e + b$$

The value of the constant b gives the intercept with F_i axis, and it had values between —4 8 and —27 7 imp sec $^{-1}$ for the seven units Of greater interest is perhaps the value of a which gives the slope of the regression line The two units of Fig 8 were selected to illustrate the extreme values of a, which ranged from 1 1 (Fig 8A) to 0 84 (Fig 8B) Accordingly, a constant amount of inhibition can cause constant reduction in firing frequency of the DSCT cells over a reasonable frequency range, or it can have an increasing effect with increasing frequency of firing It should be pointed out that there was no simple relationship between the values of the two constants a and b in the present limited material There was for instance no suggestion that a more powerful inhibition giving a large value of b was associated with a small value of the slope constant a

However, comparing the values of the slope constant with the effect of the inhibition on the variability, histogram shape and serial dependency a significant correlation appears to be present even in the present small material The units with small values of the slope constant were the ones with a reduced variability and a decreased serial dependency during inhibition The unit of Fig 8B is the unit illustrating increased regularity in Fig 3 and decreased serial dependency in Fig 7 (x) Similarly the unit illustrating increased regularity in Fig 4B had a slope constant of 0 88 The unit of Fig 8A on the other hand retained its firing pattern during inhibition (Figs 2, 4A and 7 o) Accordingly the reduced variability the reduced serial dependency and slope constants less than one appear to be a consequence of the same inhibitory mechanism

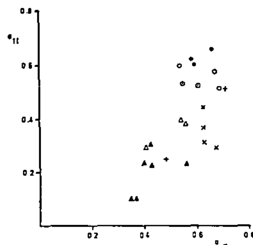


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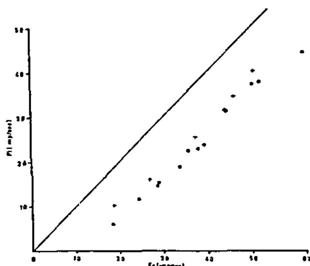


Fig 10 Comparison of effects of cortical inhibition o and GS inhibition + Data from same unit as Fig 9 Same type of plot as Fig 8 Slopes of least square linear regression lines, 0.96 for cortical inhibition, 0 93 for GS inhibition

concentrate on certain additional observations on inhibition observed in the DSCT Ia units

Stimulation of post cruciate gyrus Hongo and Okada (1967) reported IPSP's in group Ia activated Clarke's column neurones following stimulation of the post cruciate gyrus of the cat. The cortical inhibition was mediated by pyramidal tract fibres, and apparently caused only by post-synaptic inhibitory mechanisms To peripheral nerve stimulation on the other hand, they obtained evidence for presynaptic as well as postsynaptic inhibitory effects We, therefore, found it of interest to compare the firing pattern during cortical inhibition with that elicited from group I afferents of peripheral nerves

In agreement with Hongo and Okada (1967) the cortical inhibition appeared to be most easily elicited from the medial region of the post-cruciate gyrus, and this was the stimulation site in the following experiments The cortex was stimulated repetitively at a rate of 100 sec^{-1} The pulse duration was usually 1 msec and the threshold intensity was about 1 mA In search for inhibitory effects the shock intensity was increased up to 5 mA These are stimulus parameters of the same order of magnitude as those employed for instance by Hearn *et al* (1962) in order to activate pyramidal tract neurones A definite reduction in firing frequency was seen for all of the 13 Ia DSCT units examined in this way Examples of the effect are presented in Fig 9 The cortical inhibition appears to be similar to that elicited from the GS nerve, as illustrated in Fig 2 Particularly important is the finding that the cortical inhibition is maintained after an initial transient overshoot throughout the period of stimulation (Fig 9) Accordingly the firing pattern during cortical inhibition can be statistically described in the steady state period

The effect of a constant cortical inhibition at various levels of excitatory drive is

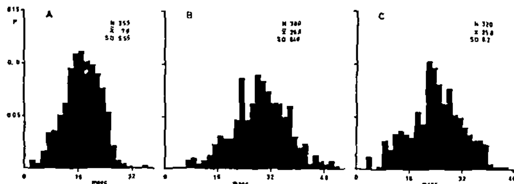


Fig 11 Interval distributions during cortical inhibition (B) and GS inhibition (C) A Interval distribution of control period immediately preceding B N, $\bar{X}$ and SD as in Fig 2

illustrated in Fig 10, in a diagram like that of Fig 8 It appears that the reduction in firing frequency was slightly greater at higher frequencies of firing The slope constant of the best fitting regression line was 0 96 The effect of group I GS nerve stimulation is shown in the same diagram from comparison In this cell the GS inhibition was slightly less effective than that elicited from the cerebral cortex The slope constant of the GS inhibition was 0 93, very nearly the same as that of the cortical inhibition

The distribution of intervals might also be similar during cortical and peripheral inhibition This is illustrated in Fig 11 The histogram obtained during the control period was as usual unimodal and fairly symmetric During cortical stimulation the firing frequency was reduced from 56 1 to 37 6 imp sec^{-1} but the distribution of intervals was still of the same shape For comparison the interval histogram obtained during GS nerve stimulation is shown in Fig 11C It is indistinguishable from that obtained during cortical inhibition

The degree of irregularity of firing might also be similar during cortical and peripheral Ia inhibition In Fig 12 the data from one unit is presented in a scatter diagram like that of Fig 5 The variation in CV was somewhat greater than usual

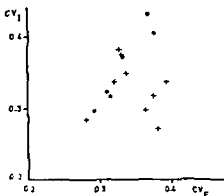


Fig 12 Coefficient of variation of interval distributions during cortical inhibition ● and GS inhibition + (ordinate) plotted against the coefficient of variation of immediately preceding control periods (abscissa) Data from same unit as Fig 9

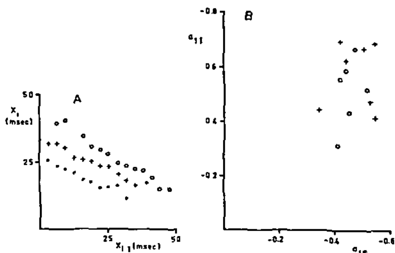


Fig 13 A Mean duration of conditional interval distributions (ordinate) plotted against the duration of preceding interval (abscissa) as in Fig 6 Group sizes of preceding intervals 3 2 [illegible]

as A Periods with firing frequencies of less than 25 imp/sec have been excluded

for this unit, but it appears that there was rather little difference between the CV of the control period and that during inhibition, and secondly that the data from cortical and peripheral inhibition are located in the same area of the diagram

Turning finally to the effect of cortical inhibition on the serial dependency between neighbouring intervals the data from the unit of Fig 10 and 12 are presented in Fig 13 Part A of this Fig is a plot of the type already employed in Fig 6 It gives the average duration of conditional intervals (ordinate) as a function of the preceding interval The frequency of firing in the control period was 56 1 imp sec ¹ The regression line then had a slope constant of —0 44 (filled circles) During cortical inhibition the mean frequency was reduced to 37 5 imp sec ¹ and the slope constant of the regression line was —0 58 (open circles) The third group of data in Fig 14A (crosses) was obtained during GS inhibition of the same cell During the GS inhibition the frequency of firing was 43 3 imp sec ¹ and this was a reduction of 13 imp sec from the preceding control period The slope constant of the conditional mean intervals was 0 47 during the inhibition These three values of the slope constant were

all within the range of variation of this particular cell All the data from this cell are presented in the diagram of Fig 13B This is a plot of the slope constant of the conditional mean intervals during inhibition (ordinate) against the same slope constant during the immediately preceding control period The values might be somewhat greater or smaller than the control values, but this is probably not significant The important finding is that the values obtained during cortical and peripheral inhibition are distributed over the same area of the graph Accordingly the serial dependency may remain essentially unchanged during cortical as well as during GS Ia inhibition

Discussion

As mentioned in the Introduction presynaptic as well as postsynaptic inhibitory mechanisms have been described operating on the Clarke's column synaptic transfer (Eccles *et al* 1963, Hongo and Okada 1967) If we accept our earlier analysis and simplified model of the excitatory synaptic coupling of the Clarke's column relay (Jansen and Walloe 1968 Walloe 1968) predictions can be made about the probable effects of the various types of inhibitory mechanisms on the firing pattern of these neurones Let us consider three different situations with a constant amount of inhibition acting on neurones at different levels of excitatory drive The first and the simplest is that of presynaptic inhibition This will cause only a reduced size of EPSPs generated by each of the primary afferent fibres With no input to the second order neurone the inhibition will have no effect and the reduction in excitation will increase directly with the intensity of the afferent input Accordingly one expects a reduced slope in the $F \sim F_p$ diagrams (*cf* Walloe 1968) In addition the presynaptic mechanism should reduce the irregularity of firing since this is primarily determined by the size of the unitary EPSPs The third predicted effect of presynaptic inhibition is a reduced serial dependency between the duration of neighbouring intervals This is because the serial dependency appears to be due mainly to the particular pattern of activity in the input fibres of the DSCT cells (Walloe 1968) and with reduced amplitudes of the EPSPs this effect would be less pronounced This particular group of effects was indeed found for some of the cells of the present material But it should be pointed out that some of the inhibitory effects that were found can not easily be explained by a presynaptic mechanism This applies particularly to the inhibition of the backgrund firing which was a regular finding in the present material

Consider next a postsynaptic inhibition which acts by hyperpolarizing the second order neurones without any concomitant shunting of the excitatory synaptic currents Eide *et al* (1969a found a linear relationship between the firing frequency and the transmembrane current for Clarke column neurone over a wide range of frequencies They also found an algebraical summation of excitatory synaptic and injected membrane currents Accordingly a steady hyperpolarizing inhibition which is caused by a steady inward membrane current in the soma will cause a constant reduction in the firing frequency over the entire range and thus give $F \sim F_p$ diagrams with a slope

of one. Furthermore, since the soma conductance is unchanged by the inhibition the size of the EPSPs will be unchanged. Therefore, one would not expect appreciable changes in the firing pattern of the neurones.

Consider finally a postsynaptic inhibition which acts predominantly by shunting the excitatory synaptic currents. The quantity of excitatory current 'lost' by the shunting would increase directly with the excitatory drive and give $F_i \sim F_e$ diagrams with reduced slopes. At the same time the size of the EPSPs will be reduced with consequences on the firing pattern as outlined for the presynaptic inhibition. In addition to these effects which presumably might be very similar to those of the presynaptic inhibition, postsynaptic inhibition with shunting will also inhibit the background firing of the cells.

Returning now to the observations presented in the Result section, it appears that the significant finding is that the DSCT neurones examined exhibited a continuous spectrum of behaviour. At the one extreme there are the units without appreciable changes in the interval-distribution and the serial dependency between intervals. These units had slopes close to one in the $F_i \sim F_e$ diagrams. The other extreme is represented by units with marked reduction in the irregularity of firing and a reduced serial dependency. This was associated with slopes significantly smaller than one in the $F_i \sim F_e$ diagrams. Intermediate types of behaviour were also observed.

From our discussion above two types of interpretation appear to be possible. The one is that the inhibition was caused by a mixture of pre- and postsynaptic inhibition in variable proportions on the various units. Alternatively, the inhibition may be purely postsynaptic with a variable degree of shunting of the excitatory currents. With the available material these two possibilities can probably not be distinguished. But on the principle of accepting the simplest model, at least as a working hypothesis, we tend to favour the second explanation. This is supported by the regular inhibition also of the background firing. Secondly, the constant occurrence of a rebound increase in firing rate after the inhibition is a point in favour of a postsynaptic mechanism. The similarity between the inhibitory effect of GS group I input and the cortical stimulation points in the same direction. As mentioned, Hongo and Okada (1967) found no evidence of a presynaptic inhibitory component efter cortical stimulation. A postsynaptic inhibition causing different degrees of shunting of excitatory currents is also easily visualized morphologically. An inhibitory synaptic input distributed over variable distances along the dendrites might well exhibit the desired properties. It has been demonstrated by Szentagothai and Albert (1955) that the primary afferent excitatory input of these neurones is located proximally on the dendrites.

Finally, some of the observations may be worth considering in a more extensive context. The high degree of linearity of the $F_i \sim F_e$ diagrams indicates that the linearity of the synaptic transfer in the Clarke column relay (Jansen *et al.* 1966) is maintained also during a constant inhibitory input. The origin of this inhibition in stretch receptors of a related muscle and its tonic nature may also be significant. The content of the signal transmitted through this subdivision of the D

garded as information on muscle length, and the observed inhibition would in the simplest case represent a constant bias on this signal so that the apparent length that the cerebellum sees is reduced Similar considerations apply to the cortical inhibition It is of interest that this is mediated by pyramidal tract fibres (Hongo and Okada 1967) which accordingly acquires a direct control over this input to the cerebellum

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The Effect of Aldosterone *in vitro* on the Active Sodium Transport and Moulting of the Frog Skin

By

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Abstract

NIELSEN, R. *The effect of aldosterone in vitro on the active sodium transport and moulting of frog skin.* Acta physiol. scand. 1969. 77. 85—94.

It is shown that aldosterone in concentrations higher than $1.5 \cdot 10^{-9}$ M induces a moult in vitro and that the moult is accompanied by characteristic changes in the potential and the short circuit current. The sequence of changes can be divided into four parts: the first constant period, the inhibition period, the spontaneous activation period and the second constant period. At a concentration of $7 \cdot 10^{-7}$ M aldosterone the inhibition starts 2—4 hrs and the activation 4—7 hrs after the addition. Furthermore it has been shown that the short-circuit current is higher in the second constant period as compared to the control skin half. During the spontaneous activation period the net sodium flux exceeds the short-circuit current by 25 %. The changes in chloride flux and sodium efflux during the moulting cycle were rather small. Both the moult and the activation of the short-circuit current were abolished by actinomycin D. A hypothesis which could explain the behavior during the moulting cycle and the activation of the sodium transport is given.

The in vitro stimulation by aldosterone of the sodium transport in the toad bladder is well established (Crabbé 1961, Porter and Edelman 1964, Sharp and Leaf 1964). The stimulation which occurs after a latent period of 40—90 min. is characterized by a gradual increase in the short circuit current. It has been shown that this stimulation is dependent upon protein synthesis (Fanestil and Edelman 1964). The action of aldosterone on the sodium transport of the frog skin has not been so fully investigated. Crabbé (1964) described experiments in which the skins of *Rana ridibunda* and *Rana esculenta* were bathed on the outside with Ringer's solution containing choline instead of sodium. After 3 hrs incubation with 10 μM d-aldosterone on the inner side of the skin, the choline Ringer's solution was replaced by normal Ringer's solution. An increased short circuit current was observed in the aldosterone treated skins as compared to the control skins.

Crabbé and De Weer (1964) have shown that the injection of aldosterone into *Bufo marinus* resulted in a stimulation of the active sodium transport across the

isolated bladder and skin Jorgensen and Larsen (1964) and Stefano and Donoso (1964) have shown "Extirpation of the pars distalis of the hypophysis inhibits shedding whereas the formation of new sloughs continues at an increased rate A slough was most often found to form within 2 or 3 days after the operation The premature formation of a slough after hypophysectomy could be further accelerated by injection of ACTH or corticosteroids (aldosterone) about 18 hrs, or a little more after the operation After such injections a complete molt could be produced within about 6—9 hours" The present experiments show that aldosterone added to the isolated frog skin produces a moult and a stimulation of the active sodium transport

Materials and methods

The experiments were performed on male and female frogs *(Rana temporaria)* The frogs were kept partially immersed in tap water at about 4° The skin was dissected from pitched animals and divided in two symmetrical halves one of which was used as a control and the *other for*
Ringer s
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chambers were used one with an area of 7 cm^2, the other with an area of 5.6 cm^2 The 5.6 cm^2 chamber was open to the exterior so that it was possible to have access to the outside of the skin which was supported on a nylon mesh (Andersen and Zerahn 1963) Radionuclides used were chloride 36, sodium 22 and sodium 24 Sodium 22 and sodium 24 were counted with a Selectronic autogamma spectrometer and the chloride 36 with a Packard liquid scintillation spectrometer using a naphthalene dioxane solution as scintillator The aldosterone (Aldocorten Ciba) was added to both sides of the skin unless otherwise stated

Result

The effect of aldosterone on the short circuit current and potential in the frog skin Fig 1A shows a typical result of adding $7 \cdot 10^{-7}$ M aldosterone to the medium bathing the frog skin while fig 1B shows the corresponding control The graphs depicting the short circuit current and the potential after addition of aldosterone are divided into four parts A—B is called the first constant period B—C the inhibition period C—D the spontaneous activation period and D—E the second constant period At a concentration of $7 \cdot 10^{-7}$ M aldosterone the inhibition started in most *cases 2—4 hrs and the activation 4—7 hrs after the addition* The initial decline in the short circuit current during the constant period is the usual decrease seen at the commencement of short-circuiting conditions and is not due to the addition of the aldosterone (*cf* Fig 1B) In other experiments in which the short circuit current was steady the addition of aldosterone produced no initial decrease In 7 out of 17 expts there was a small but significant increase in the short circuit current starting 40—90 min after the addition of the aldosterone (Fig 2) 3 of these 7 expts did not show any subsequent inhibition and activation period With a lower concentration of $1.5 \cdot 10^{-9}$ M (6 expts) there was normally no clear indication of when the in*hibition period started and the activation commenced* 7—12 hrs after addition of aldosterone Four attempts to demonstrate activity at 7 · 10[illegible] M were unsuccessful Experiments with a higher concentration of $7 \cdot 10^{-6}$ M (4 expts) showed the same

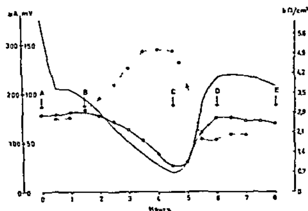


Fig 1A Effect of aldosterone on the short-circuit current and potential across the frog skin
At zero time aldosterone was added on both sides to give a concentration of 7 10⁻⁹M

——— short-circuit current (μA/7 cm²)
○—○ potential (mV)
● ● resistance (ohm cm²)

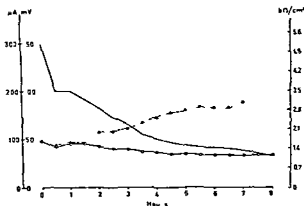


Fig 1B Control skin half without added aldosterone symbols as in Fig 1A

effect as those performed with 7 10 M The concentrations which provide minimal and maximal response are therefore about 1 5 10⁻⁹ M to 7 10 M respectively The hormone elicited its effect both from the inside and the outside

Effect of mechanical action During the latter part of the inhibition period the skin is very sensitive to the mechanical action of changing the Ringer s solution or applying a pressure difference Fig 3 shows the result of applying a pressure of 50 cm of water for 20 sec to the inside of the skin Immediately a large increase in short circuit current was observed in the aldosterone treated half skin whereas there was little change when a pressure difference was applied to the control This effect was absent when a pressure difference was applied in the first constant period or in the first part of the inhibition period Further investigation showed that the same or even a higher activation could be obtained if the stratum corneum were removed (Fig 4A) Experiments with an open chamber showed that this effect was due to the removal of the stratum corneum and not to the mechanical disturbance resulting from draining and refilling the chamber The stratum corneum

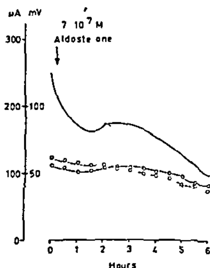


Fig 2 Early activat on by aldosterone in the short circuit current and potential observed in 7 out of 17 expts

——— short circuit current aldosterone treated skin
O———O potential aldosterone treated skin
short-circuit current control skin half
O O potential control skin half

was removed by gentle rubbing of the skin with a cotton wool tampon and it slipped off rather easily in the aldosterone treated skins whereas it could not be removed in the control half (Fig 4B) Again no effect was observed in the first constant period or in the first part of the inhibition period attempts to remove the corneum during these periods were unsuccessful and were always accompanied by a decrease in the potential In only a few experiments was there any activation of the short-circuit current and it was of a small magnitude

The effect of aldosterone on the sodium flux To ascertain whether the changes in the short-circuit current following aldosterone treatment were due to an effect on the active transport or on the passive flux or on the combination of both measurements were performed using the double labelling technique of Levi and Ussing (1949) Two series of experiments were performed In one the stimulation was allowed to proceed spontaneously (Table I) in the other the stimulation was induced by removal of the stratum corneum (Table II) There was good agreement between the net sodium flux and the short-circulit current during the inhibition period (*B—C* and the second constant period (*D—E*) However during the ac tivation period C—D there was a difference between the two series of experi ments When the activation was spontaneous the short-circuit current was abo it 25 % higher than the net sodium flux (Table I) When the activation was induced by the removal of the tratum corneum there was a tendency for the short-circuit current to be lower than the net sodium flux (Table II) even though the sodium efflux was doubled by the treatment *cf* Table I and II The cause of the d s repancy between th net sodium flux and the short-circuit current during the a ation period w s sh rt lived because good agreement between the two m rements was f d again during the one hour period following activation

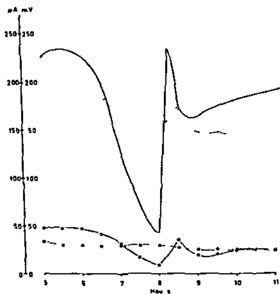


Fig 3

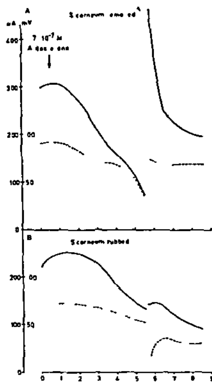


Fig 4

Fig 3 Effect of applying a pressure of 50 cm of water to the inside for 20 sec
At zero time aldosterone was added on both sides to give a concentration of 7 10 ⁷M Pressure applied 8 hrs after the addition of aldosterone when the aldosterone treated skin was in the inhibition period

——— short-circuit current aldostorene treated skin
O——O potential aldosterone treated skin
short circuit current control skin half
O -O potential control skin half

Fig 4A Effect of removing the stratum corneum At zero time aldosterone was added to both sides to give a concentration of 7 10 ⁷M The stratum corneum was removed 5 ½ hrs after the addition of aldosterone

——— short circuit current (μA/7 cm²)
O——O potential

Fig 4B Control skin half without added aldosterone symbols as in Fig 4A

Table III shows the changes in the sodium efflux during the first constant period and the first and the second half of the inhibition period in 6 half skins It appears that the efflux was rather constant during this period

The effect of aldosterone on the chloride flux In the previous section it was shown that the short circuit current was higher than the net sodium flux in the spontaneous activation period This could be due to an active transport of chloride from the inside to the outside since Koefoed Johnsen Ussing and Zerahn (1952) have shown

TABLE I Comparison of the sodium flux and the short-circuit current across the frog skin with spontaneous activation following aldosterone treatment

Period	influx	efflux	A Mean net sodium flux	B Mean short-circuit current	B—A	±S E	p
			$\mu A/7\ cm^2$				
(B_1—C_1)	118 6	11 9	106 6	101 7	— 4 9	3 6	$0.2 < p < 0.25$
(B_2—C_2)	62 1	9 9	52 2	49 1	— 3 1	2 4	$0.1 < p < 0.2$
(C—D)	94 0	15 1	79 0	97 7	18 4	1 9	$p < 0.001$
(D—E)	169 8	11 8	158 0	155 6	— 2 4	2 2	$0.3 < P < 0.4$

The influx was measured by means of Na-22 and the efflux simultaneously with Na-24, the short-circuit current was measured and recorded automatically The mean results from 6 expts are presented, in which each period was about 1 hr B_1—C_1 first half of inhibition period, B_2—C_2 second half of inhibition period, C—D activation period, D—E constant period For an acutal pettern of an experiment see fig 1A

TABLE II Comparison of the sodium flux and the short-circuit current across the frog skin after the mechanical removal of the stratum corneum following aldosterone treatment

Period	Influx	efflux	A Mean net sodium flux	B Mean short-circuit current	B—A	± S E	p
			$\mu A/7\ cm^2$				
Activation	301 7	39 0	262	238	—24	14 2	$0.1 < p < 0.2$
Constant	248 0	29 0	218	221	3	4 3	$0.3 < p < 0.4$

The influx was measured by means of Na-22 and the efflux simultaneously with Na-24 The short circuit current was measured and recorded automatically The mean results from 6 expts are presented, in which each period was about 1 hr For an actual pattern of an experiment see Fig 4 The isotopes were added immediately after the corneum was removed The first samples were taken 15 min after the addition of the isotopes

that the glands of the skin actively liberate chloride to the outside when the skin is treated with adrenaline Six experiments were therefore performed in which the chloride influx and efflux were measured separately on symmetrical half-skins during aldosterone treatment with subsequent spontaneous activation (Table IV) The chloride permeation was constant during the first constant period (A—B) and the inhibition period (B—C) there was in five of the experiments an increase in the chloride permeation during the activation period (C—D) but influx as well as

TABLE III Effect of aldosterone on sodium efflux during the first constant period and the inhibition period

Period	Constant first	Inhibition first half	Inhibition second half
		μA/7 cm²	
	11 5	10 7	14 7
	8 2	7 5	
	11 5	11 3	12 6
	10 7	10 8	11 9
	13 1	12 6	16 1
	27 2	23 4	22 5

TABLE IV Effect of aldosterone on chloride fluxes during spontaneous activation

Exp no	Influx				Efflux			
Period	A—B	B—C	C—D	D—E	A—B	B—C	C—D	D—E
				μA/7 cm²				
1		13 4	13 9			8 8	11 0	
2		26 3	30 0	33 0		11 0	18 8	15 0
3	11 8	12 6	16 1	14 5	19 3	18 2	22 0	22 8
4	12 1	7 2	24 7	25 7	20 6	21 4	27 3	23 3
5	10 7	11 5	15 5	19 3	18 5	15 3	23 8	29 4
6	71 8	79 9	49 6	68 8	72 6	87 8	64 9	62 9

The chloride influx and efflux were measured simultaneously on symmetrical halves of a frog skin, during aldosterone treatment

A—B first constant period C—D spontaneous activation period
B—C inhibition period D—E second constant period

efflux was equally affected Thus there was no net movement of chloride during the activation period which would account for the discrepancy of 18 7 μA/7 cm observed between the net sodium flux and the short-circuit current

Effect of actinomycin D on the aldosterone treated frog skin To examine whether the aldosterone effects was dependent on protein synthesis 6 expts were performed in which two skin halves were incubated with $7 \cdot 10^{-}$ M aldosterone, but one half had in addition 6 μg/ml of the protein synthesis inhibitor actinomycin D The actinomycin D abolished both the inhibition period which commenced after about 3 hrs and the activation period which commenced after five hours (Fig 5) in the control half

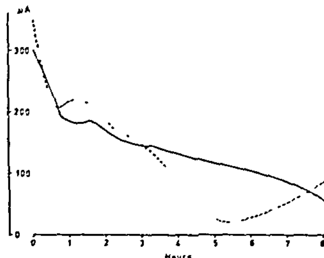


Fig. 5. Effect of actinomycin D on the aldosterone treated frog skin. ——— short-circuit current ($\mu A/7\ cm^2$). At zero time aldosterone and actinomycin D were added on both sides to give concentrations of $7 \cdot 10^{-7}$M and 6μg/ml respectively. • • • • • • • short-circuit current ($\mu A/7\ cm^2$). At zero time aldosterone was added on both sides to give a concentration of $7 \cdot 10^{-7}$M.

Discussion

It appears from the above observations that aldosterone induces a "moult" in vitro, i.e. the separation of the stratum corneum from the stratum granulosum, and that the moult is accompanied by characteristic changes in the electrical potential and the short-circuit current, one of which is a late activation of the short-circuit current. The fact that both these processes occur only after a latent period and are abolished in the presence of actinomycin D indicates that they are dependent on protein synthesis. A comparison between Fig. 1A and Fig. 1B shows that the short-circuit current is higher in the second constant period (D—E) in the aldosterone treated skin than in the control. The same is seen from Fig. 3 where the activation is induced by pressure treatment. The experiment shown in Fig. 4 indicates that the transport system already is activated when the skin is in the inhibition period, since the removal of the stratum corneum immediately causes an activation of the short-circuit current and the potential. A hypothesis to account for these observations has to provide answers to the following questions.

1. Why do the short-circuit current and the potential exhibit the pattern shown in Fig. 1A? The experiments show that the inhibition is not due to an increase in passive fluxes, since the sodium efflux and the chloride fluxes are constant during the first constant period and the inhibition period (Table III and IV). The inhibition must therefore be due to a specific action on the active sodium transport.

2. Why is there a discrepancy between the short-circuit current and the net sodium flux during the spontaneous activation period (Table I, C—D)? From Table IV it is seen that this discrepancy is not due to an active transport of chloride.

Why does the rem[illegible]al of the corneum induce a sharp rise in the short-circuit cur[illegible] and the potent[illegible] (Fig. 4)? The following working hypothesis would ex-

plain these observations During the first constant period an enzyme which attacks the material between the stratum corneum and the stratum granulosum is formed and released During the break down a subcorneal space is formed (Voute Nielsen and Ussing 1968) The space contains a compound which inhibits the active sodium transport this postulate is supported by the fact that the active transport is activated immediately if the cornified layer is stripped off when it would be expected that the inhibitor would rapidly diffuse away The inhibitor could be a product of the brokendown cement material The spontaneous activation could then be due to the gradual diffusion away or chemical disentegration of the inhibitor The discrepancy between the net sodium flux and the short circuit current during the spontaneous activation period could be explained by the fact that the samples are not taken during steady state conditions It has been shown by Hoshiko and Ussing (1960) and Andersen and Zerahn (1963) that it needs 0 3 to 1 5 μeq sodium/7 cm to bring a skin from an unlabelled state to the labelled state During the latter part of the inhibition period the influx of sodium 22 through the skin is very low, and the skin would be in a nearly unlabelled situation with respect to sodium 22 if the influx barrier as generally accepted is close to the outside of the skin During the spontaneous activation period the sodium 22 influx increases which would bring the skin from a nearly unlabelled state to a labelled state Thus the discrepancy observed (0 6 μeq/7 cm²) is used to bring the skin from a nearly unlabelled form to a labelled form

The cycle is finished when the skin is in the second constant period The corneum has now become so leaky due to the action of the enzyme that the ions diffuse easily through it and the skin is back in its normal transport situation The above working hypothesis explains the qualitative behavior during the moulting cycle but it does not explain why the short circuit current is increased in the second constant period as compared to the control half A possible explanation would be that the diffusion of sodium through the corneum and the material between the corneum and the stratum granulosum was the rate limiting step The activation observed would then be a consequence of the breakdown of this material and the corneum during the moult However in 7 out of 17 skins (Fig 2) there was an increase in the short circuit current 40—90 min after the addition of aldosterone as in the toad bladder These skins were probably skins which had just moulted since it was observed in 4 of the 7 that the corneum slipped off during mounting of the skin The results of these 7 expts may indicate that aldosterone has two effects on the frog skin 1 it activates the sodium transport in a unknown manner 2 it induces a moult However the activation could be due to an additional effect on the outermost border of the stratum granulosum by the moult enzyme or enzymes If it is assumed that an early activation normally is superimposed on the inhibition during the moult a higher short circuit current would be expected in the aldosterone treated skins when the inhibitor concentration was low enough not to abolish the activation A low inhibitor concentration could be due to the lack of a corneum or to a corneum which is rather permeable for the inhibitor so that this does not pile up Another possibility

would be that the amount of precursor to the inhibitor is low. One of these or a combination of both possibilities may be the case in recently moulted skins. However it is not yet possible from the experimental data to distinguish between these hypotheses.

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The Heat Regulation of the Human Body

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Abstract

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On the basis of a comparison between calculated surface temperature of a physical model and the results from experiments on human subjects it is concluded that the heat loss by radiation and convection plus the uncontrolled evaporation through the skin surface is equal to the heat flow by conductance through the tissues, and the amount of heat given off by evaporation of the regulated sweat secretion is equal to the amount of heat which is transported by the blood stream from the interior to the skin surface The only regulation necessary for the maintenance of the total heat balance, therefore is a thermostatic control which can regulate the heat transporting blood stream from the interior to the skin surface as well as the secretion of sweat from the glands

It is usually assumed that the skin temperature of man is regulated by a vasomotor control mechanism whereby a decrease in temperature produces an additional decrease of the skin temperature This would give an additional decrease of the heat loss and thereby make it easier to maintain the internal temperature at 37° C

Whilst reexamining the temperature curves shown in Fig 1, originating from experiments with a clothed subject by Nielsen and Pedersen (1952), it became apparent that these curves resembled curves which show the temperature in different layers of insulating material on an inner surface at constant temperature

Experiments have shown that evaporation at constant heat production increases linearly with skin temperature and, at constant skin temperature, evaporation increases in proportion to heat production, whereas skin temperature and heat production are independent of one another It has not been possible to give an adequate explanation of the mechanism of the regulation of sweat secretion or of the details of heat regulation This suggested that it would be useful to examine to what extent it is possible to give an explanation of the heat regulation of the human body based on physical principles

In order to examine the validity of the original assumption that skin temperature is not regulated by vasomotor regulation the cylinder shown in Fig 2 will be considered

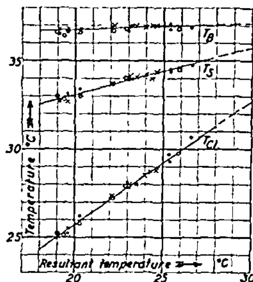


Fig 1 The rectal temperature, the skin temperature and the clothing temperature in relation to the resultant or operative temperature

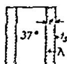


Fig 2 Cylinder with wall thickness e m, heat conductance λ kcal/m²hr °C, surface temperature t_s C and inner temperature 37° C

The heat flow through the wall of the cylinder is $\frac{\lambda}{e}(37-t_s)$ kcal/m²hr and this value is equal to the heat loss from the surface $k(t_s-t_0)$ kcal/m²hr, where t_0 is the ambient temperature and k is a coefficient, which for vertical surfaces in still air is $k=4.71+2.2(t_s-t_0)^{\frac{1}{4}}$. It is therefore possible to calculate the surface temperature of the cylinder when its thickness and heat conductance are known. Since in this case, however, we want to compare the surface temperature of the cylinder with the skin temperature of the human being under equal external conditions, it is necessary to introduce a correction for the cylinder corresponding to the unregulated evaporation through the skin, which is about constant at 20 kcal/m²hr (*cf* Figs 4—7). The surface temperature of the cylinder can be calculated from $\frac{\lambda}{e}(37-t_s)=k(t_s-t_0)+20$ kcal/m²hr. The curve no 1 in Fig 3 shows the relation between the surface temperature and the temperature of the environment, when $\frac{\lambda}{e}=20$ kcal/m²hr and the cylinder is placed in a room with still air. Curves no 2 and no. 3 show the surface temperature when $\frac{\lambda}{e}=20$ kcal/m²hr C and the cylinder placed in air with a velocity of 50 and 100 cm/sec respectively. The radiation constant is 4.71 kcal/m²hr C and the convection constant valid for air in movement has been determined by Winslow, Gagge and Herrington (1939) to be $C=1.0\sqrt{V}$ kcal/m²hr C where V is the air velocity in cm/sec. For air movements of 50 and 100 cm/sec the total heat loss constants involving radiation as well as convection are, therefore $k=11.8$ kcal/m²hr C and $k=14.7$ kcal/m²hr °C, respectively.

As mentioned above the curves no 2 and no 3 in Fig 3 are calculated for $\frac{\lambda}{e}=20$ kcal/m²hr °C and for air velocities of 50 and 100 cm/sec respectively, but curve no 2 also corresponds with still air and $\frac{\lambda}{e}=16$ whereas curve no 3 also corresponds with still air and $\frac{\lambda}{e}=13.5$ kcal/m²hr C

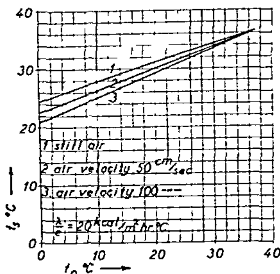


Fig 3 The relationship between calculated surface temperature t_s and the *operative temperature* t_o *for a cylinder* with an inner temperature of 37° and a constant evaporation of 20 kcal/m²hr from the surface

Fig 4 and Fig 5 are based on experiments by Winslow and Gagge (1941) The unclothed subjects carried out light work on a bicycle ergometer, pedalling rate 48 rds/min, air velocity 5—8 cm/sec (Heat production Q kcal/m²hr)

Comparison of the measured skin temperatures from Fig 4 and 5 with the calculated surface temperatures of the cylinder as shown in the curves in Fig 3, shows

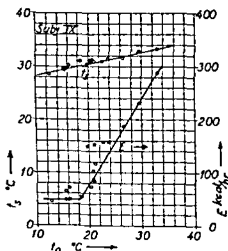


Fig 4

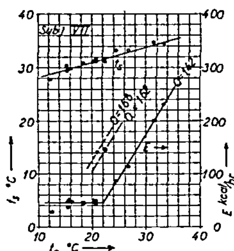


Fig 5

Fig 4 The relationship between skin temperature t_s and evaporation E and the environmental temperature t_o Subject IX height 1 72 m weight 62 5 kg area 1 60 m² Q=171 kcal m²hr T_R – 37 6° C.

Fig 5 The relationship between skin temperature t_s and evaporation E and the environmental temperature t_o Subject VII height 1 88 m weight 79 kg area 1 9? m², Q=142 kcal m²hr, t_R = 37 6 C

that these corresponding temperatures are in good agreement with one another The skin temperature curve in Fig 5 is practically identical with curve 1 in Fig 3

The comparisons made between the skin temperatures and the calculated surface temperatures show that the skin temperature of the human body, at equal environmental conditions, vary in the same way as the surface temperature of a physical body with an adequate insulation and with an inner temperature of 37° C

From Fig 4 and 5, where also the evaporation curves are drawn, it will be noted that the rapid increase in the value of E at room temperatures from about 18° C and about 22° C, respectively, does not influence the skin temperature curves, and the level of the skin temperature is not influenced by the level of the evaporation

As to evaporation and skin temperature Winslow and Gagge in their paper (1941) state

> Evaporative regulation in the case of the working subject operates so effectively that skin temperature is held remarkably constant actually being lower—at a given t_o — for the working than for the resting body With increasing metabolism at a given t_o the skin temperature of the trunk actually falls

One, therefore, can conclude that *the skin temperature remains uninfluenced by the vasomotor reactions and the changes in evaporation of sweat*

If the cylinder with $\frac{\lambda}{c} = 20$, shown in Fig 2, is supplied with a certain amount of heat and the inner temperature is maintained at 37° C, the supplied heat must be given off from the surface of the cylinder Thus if the heat loss coefficient is k=13 4 kcal/m²hr °C (corresponding to an air velocity of 75 cm/sec), the heat production 60 kcal/m²hr and the heat loss by evaporation 20 kcal/m²hr, we have

$60 = 20\ (37 - t_s)$, from where $t_s = 34\ 0°$ C

and $60 = 13\ 4\ (34\ 0 - t_o) + 20$, from where $t_o = 31\ 0°$ C

This means that a heat production of 60 kcal/m²hr exactly will cause equilibrium when the operative temperature is 31 0° C, the skin temperature—to return to the human being—is 34 0° C and with the air velocity as mentioned above

If the environmental temperature, during the stated conditions, rises above 31 0° C the skin temperature also will rise, but to a smaller degree, and, therefore, the amount of heat (60 kcal/m²hr) can only be given off by extra evaporation equal to the decrease in the heat loss by radiation and convection This means that this point of equilibrium is that skin temperature at which regulated evaporation begins to come into action as a heat controlling factor For each value of the heat production there is such a *point of low equilibrium* It is possible to calculate these points of equilibrium when the value of $\frac{\lambda}{c}$ for the exterior tissues is known

This value of $\frac{\lambda}{c}$ for the conductivity of the tissues can be determined by experiment

Fig 4 shows that regulated evaporation (the steep part of the evaporation curve)

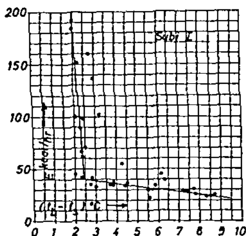


Fig 6

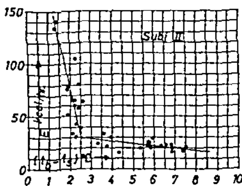


Fig 7

Fig 6 Evaporation in relation to the difference between rectal and skin temperature Subj I, 2 13 m², Q=45 6 kcal/m²hr

Fig 7 Evaporation in relation to the difference between rectal and skin temperature Subj II, 1 49 m², Q=49 0 kcal/m²hr

begins at $t_0=18°$ C which corresponds to $t_s=30\ 0°$ C As the heat production at these experiments was 171 kcal/m²hr and the rectal temperature was 37 6° C, the conductivity can be calculated from

$$\frac{\lambda}{e}=\frac{171}{37\ 6-30\ 0}=22\ 5\ \text{kcal/m}^2\text{hr °C}$$

From Fig 5, in a similar way, appears

$$\frac{\lambda}{e}=\frac{162}{37\ 6-31\ 4}=22\ 9\ \text{kcal/m}^2\text{hr °C}$$

Fig 6 and Fig 7 show evaporation in relation to the difference between rectal and skin temperature for two unclothed resting subjects The experiments were carried out by Winslow, Herrington and Gagge (1937) Subject I, who was of the pyknic type, had a heat production of 45 6 kcal/m²hr, and subject II, who was of the leptosomic type, had a heat production of 49 0 kcal/m²hr It appears from Fig 6 and Fig 7 that the transition from the slight ascent of the curve (evaporation without regulation) to the steep ascent of the curve (regulated evaporation) takes place at a temperature difference of about 2 4° C and 2 5° C, respectively Based on these values follows for subject I $\frac{\lambda}{e}=\frac{45\ 6}{2\ 4}=19\ 0$ kcal/m²hr °C, and for subject II $\frac{\lambda}{e}=\frac{49\ 0}{2\ 5}=19\ 6$ kcal/m²hr °C

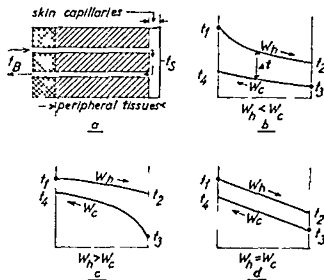


Fig 8 Illustration in principle of the heat transport by the blood stream

The value of $\frac{\lambda}{e}$ will probably depend on the amount of fat in the tissues The values of $\frac{\lambda}{e}$, mentioned above, are based on heat productions which are not corrected for heat loss by evaporation from the lungs, and, therefore, the calculated values are a little too high For tissues containing about 70 per cent of fluids the conductivity may be estimated to $\lambda = 0\ 42$ kcal/mhr °C which will result in a mean thickness of the tissues, corresponding to the temperature difference $(37-t_s)$ of 0 021 m for $\frac{\lambda}{e} = 20$

It was mentioned earlier in this paper that a rise of the skin temperature above the low equilibrium point coresponding to a given heat production involved that the dissipation of the produced amount of heat could be effected only by supplementing the heat loss by radiation and convection plus the unregulated evaporation with an additional evaporation of sweat from the skin surface Furthermore, the amount of heat produced in the interior cannot be transported to the skin solely by the conductivity of the tissues but it is necessary to utilize circulation of blood through the exterior tissues This circulation must be such that the full amount of heat can be transported from the interior to the surface by conduction plus circulation

We will therefore investigate how this heat transport by the blood stream through the peripheral tissues to the skin capillaries takes place

Fig 8a shows a model of the blood stream system from the inner core to the skin where the capillaries in the figure are represented by a narrow box Fig 8 b—d show the variation of temperature along the tubes by counter current The hot stream has the indication h and the cold stream has the indication c Wh signifies

the water value (weight times specific heat) of the hot stream, and Wc the water value of the cold stream

The transmission of heat q from the hot to the cold stream is

$$q=c \cdot F \cdot \Delta t \text{ kcal/hr}$$

where c is the heat transmission coefficient for the tissues between the tubes, and F is the transmission area, while Δt is the mean difference between the temperature curves

From Fig 8 d it appears that the temperature difference is constant when the two counter currents have the same water value A similar situation occurs in the human body (*cf* Fig 8 a) This means that the amount of heat Q kcal/m²hr which is transported from the interior by the blood stream B l/m²hr is

$$Q=B \cdot \Delta t \text{ kcal/m}^2\text{hr}$$

This, necessarily, must also equal the amount of heat which is transported by the blood stream to the skin surface from where it has to be given off if the temperature equilibrium is to be maintained As the amount of heat which is given off from the hot blood stream to the peripheral tissues is exactly the same as the amount received by the cold blood stream, there is no real heat interchange between blood streams and tissues which merely serve as intermediate conductive material between the hot and the cold stream

The cooling of the blood stream ($\Delta t=t_2-t_3$), which is necessary for the heat transport, takes place in the skin capillaries and is caused by the evaporation of sweat

On the other hand, the transmission area F, the conductivity c of the tissues as well as the blood flow B will influence the possible value of Δt This is shown in Fig 9 where Δt is related to t_3 (*cf* Fig 8 d) when $t_1=37.0°$ C and c F=100 n kcal/°C hr and the circulation of blood through the skin is as stated on the lines drawn in Fig 9

It is not possible from Fig 9 to calculate the value of Δt, which is equal to the cooling of the blood stream through the skin capillaries, but it is possible, though, to estimate how the value of Δt varies with the amount of blood and with the value of c F For instance it appears from Fig 9 that t_3—30° C results in $\Delta t=2°$ C when B= =40 n l/m²hr As n is an arbitrary number, it appears from this that, for instance, twice the amount of blood is required to double the amount of heat transmission from hot to cold vessels when Δt is constant

The value of c F for the human body is not known F is the total surface of the vessels which carry the hot blood stream to 1 m² of skin surface through the exterior tissues in a layer of about 2 cm thickness, and $c=\frac{\lambda}{d}$, where λ is the conductivity of the tissues and d is the mean distance between the surface of the hot and the cold vessels

From above it is possible to set up the following equations for the heat balance of the human body

$$Q-\frac{\lambda}{e}(t_B-t_s)+B \cdot \Delta t \qquad (1)$$

$$Q = k(t_s - t_o) + E_u + F_{re} \tag{2}$$

in which the significations of the symbols are as follows

Q the amount of heat produced in kcal/m²hr minus the respiration heat
λ the conductivity of the periphery tissues in kcal/m²hr °C
e the thickness in m of the layer corresponding with the temperature difference $(t_{II} - t_s)$
t_{II} the temperature of the interior in °C
t_s the skin temperature in °C
B the blood stream in l/m²h
Δt the temperature difference in °C between hot and cold blood stream
t_o the temperature in °C of the environment (operative temp)
k the heat loss coefficient in kcal/m²hr °C
E_u the heat loss by unregulated evaporation in kcal/m²hr
E_{re} the heat loss by regulated evaporation in kcal/m²hr

It has been demonstrated above that the skin temperature is uninfluenced by the regulated evaporation of sweat which starts at the low balance point at which $\frac{\lambda}{e}(t_{II} - t_s) = k(t_s - t_o) + E_u$ Increase in the environmental temperature results in a decrease in heat loss by radiation and convection and this decrease is instead given off by evaporation Therefore the equation

$$\frac{\lambda}{e}(t_{II} - t_s) = k(t_s - t_o) + E_u \tag{3}$$

is valid at any environmental temperature

Hence also follows that

$$E_{re} = B \Delta t \tag{4}$$

Eq 1 expresses how the heat produced is transported from the interior to the skin surface by conduction and by transportation by the blood stream

Eq 2 expresses how the heat produced is dissipated from the skin surface, partly by radiation and convection and partly by evaporation

Eq 3 expresses that the heat transmission by conductance through the tissues is equal to the heat loss by radiation and convection to the environment plus the unregulated evaporation through the skin

From this follows eq 4, which expresses that the heat loss by regulated evaporation of sweat is equal to the amount of heat which is transported to the surface by the blood stream

Now we will investigate the way in which the heat balance may be regulated If the organism is constituted in such a manner that the inner temperature is maintained at a constant value, it seems most likely that the heat transporting blood stream is thermostatically regulated by a center in the brain from where nervous impulses operate the vasomotory vessels in the skin This must be done in such a way that the blood stream brings exactly the right amount of heat from the interior to the skin surface This indicates that when the inner temperature is raised, the capillaries must be opened somewhat, and when the inner temperature is decreased the capillaries must tend to close In this way the amount of heat which cannot be led away solely by conductance will be transported by the blood stream, and thus the inner temperature will be maintined at a constant level

Furthermore, it may be assumed that the secretion of sweat is controlled by the heat regulating center in the brain parallel to the control of the blood stream through the skin Since the evaporation of sweat from the skin causes the cooling of the blood in the capillaries, we have

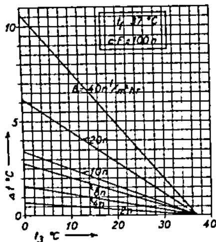


Fig 9

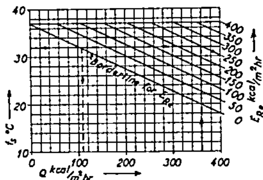


Fig 10

Fig 9 The relationship between Δt and t_s for heat transmission by countercurrent $t_i = 37.0°$ C, c F = 100 n kcal/h° C, B = a n l/m²hr

Fig 10 Skin temperature in relation to heat production at low equilibrium, and evaporation in relation to heat production and skin temperature

$$\Delta t = \frac{E_{re}}{B}$$

If Δt is constant, the amount of heat transported from the interior by the blood stream is proportional to the amount of blood, and also the sweat secretion and the evaporation are proportional to the amount of blood Whether Δt is constant or not, both the amount of heat transported by the blood from the interior and the heat given off by evaporation of the sweat secretion will be equal to B Δt kcal/m²hr

The only regulation necessary to the maintenance of the total heat balance, therefore, is a thermostatic control which can regulate the heat transporting blood stream from the interior to the skin surface as well as the secretion of sweat from the glands

Because the supply of heat from the blood stream to the skin capillaries, B Δt kcal/m²hr is essentially uninfluenced by the skin temperature and is determined by the amount of blood and the size of Δt—which again is determined by the evaporation—, the skin temperature will be fixed in accordance with eq 3 Furthermore, the amount of heat which is carried along with the blood stream is given off from the surface solely by evaporation of sweat which, in practice, is also uninfluenced by the temperature of the skin

Fig 9 shows, however, that the size of Δt, at a given blood flow, decreases rapidly with rising t_s—and, therefore, with rising skin temperature If the cooling caused by the evaporation is greater than that corresponding to Δt for the blood stream at high skin temperature, a cooling of the skin tissues will result This is due to the temperature of the hot blood stream at the beginning of the skin capillaries being

only slightly higher than the skin temperature when the difference between the body temperature and the skin temperature is small The cooling effect from evaporation will, therefore, involve a decrease of the skin temperature This is most likely the reason why the skin temperature curve often has a sharp bend at about 34 5° C in rest experiments

When the thermostatic mechanism of regulation was discussed, it was assumed that the organism maintains a constant core temperature This, however, is only the case at rest, whereas the inner temperature during work conditions will vary with the work loads Nielsen (1938) demonstrated that the rectal temperature during work rose to an equilibrium level independent of the environmental temperature and solely determined by the rate of work Marius Nielsen concluded that the rise of temperature was not due to an insufficient heat regulation, but that the organism adjusted the temperature level in accordance to the work rate This relation between work and inner temperature has later on been confirmed by Bodil Nielsen and Marius Nielsen In these experiments the rectal temperature as well as the temperature deep in esophagus was measured and the experiments showed that the esophageal temperature varied in a similar way as the rectal temperature in relation to the work rate

When the inner temperature of the body varies with the work rate, the organism must be able to readjust the regulating thermostat to a new level according to the rate of work and the heat balance must be regulated in accordance to this new level

It was mentioned earlier in this paper that each amount of heat production corresponds to a certain skin temperature at low equilibrium, and at this point the circulation of blood and the secretion of sweat start as heat regulating factors Below these points, therefore, it is impossible to maintain equilibrium, and a lowering of the ambient temperature, and with that a lowering of the skin temperature, will result in a decrease of the inner body temperature

Fig 10 shows the skin temperature in relation to the heat production at low equilibrium Below the border line in the figure, the heat balance cannot be maintained and in this area, therefore, the body temperature will be below the fixed setpoint The border line is drawn in accordance with $\frac{\lambda}{e} - 20$ kcal/m²hr °C, $t_B = \left(36\ 9 + \frac{1\ 1\ Q}{380}\right)$ °C and in still air Q signifies the heat production in kcal/m²hr

Above the border line the blood circulation as well as the secretion of sweat will be related and the heat balance will be in equilibrium

At the upper limit the heat balance will be restricted since the blood flow and the secretion of sweat can no longer be increased In humid and hot climates the evaporation of sweat will be limited too in accordance with the difference of water vapor pressure between skin and surroundings, but as long as the sweat does not run or drop off, the full evaporative power will be maintained

Fig 10 shows too the relationship between evaporation and heat production and skin temperature For instance it appears that a heat production of 360 kcal/m²hr and a skin temperature of 32 0° C correspond to a regulated evaporation of 250

kcal/m²hr whereas radiation plus convection plus unregulated evaporation amount to 110 kcal/m²hr This heat loss corresponds to the heat production at the point of low equilibrium at $t_s = 32.0°$ C

The account above illustrates how the heat regulation of the human body may take place and this theory relates the heat production and the heat loss under varying work loads and environmental temperatures

I am indebted to Professor D Ph Marius Nielsen and to Amanuensis Bodil Nielsen for guidance in physiological questions

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Reciprocal Control of Spontaneous Activity and Reflex Effects in Static and Dynamic Flexor γ-motoneurones Revealed by an Injection of DOPA

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Abstract

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Single γ-efferents to flexors have been studied in the spinal unanesthetized cat before and after an intravenous injection of DOPA. The γ-efferents could be classified in two groups, one with a high level of spontaneous activity that was decreased after an injection of DOPA, and another with a low level of activity that was enhanced after DOPA. These data together with differences in reflex behaviour between the two types of γ motoneurones provided a basis for a comparison with the deduced properties of dynamic and static γ motoneurones obtained from studies of spindle afferents with primary and secondary endings It could be concluded that the former type is identical with dynamic and the latter with static γ motoneurones. Both dynamic and static neurones could be reflexly activated in the long latency flexor reflex occurring after DOPA Likewise both types of γ motoneurones were activated in the short latency flexor reflex, the dynamic more effectively before and the static more effectively after DOPA. The results suggest that descending noradrenergic fibres control reflex activation and the spontaneous activity of γ motoneurones to flexors in a reciprocal fashion

The influence of an intravenous injection of DOPA on the transmission in the spinal cord has been studied extensively (Andén et al. 1966a, Andén, Jukes and Lundberg 1966b Jankowska et al 1967a b) The action of DOPA is blocked if it is preceded by an injection of substances blocking the synthesis of noradrenaline or its precursor dopamine from DOPA thus relating the effect of DOPA to the synthesis of noradrenaline (Andén et al 1966b Jurna and Lundberg 1968) For this and other reasons it has been postulated that an intravenous injection of DOPA acts by an increased synthesis and liberation of noradrenaline from the noradrenaline containing terminals of descending fibres (Andén et al 1966a, b Jurna and Lundberg 1968) Such fibres originating from cells in the lower brain stem (Dahlström and Fuxe

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1965), were described by histochemical methods by Carlsson *et al* (1964) They also showed that no other neurones in the spinal cord contain noradrenaline The facts that noradrenaline is stored in large amounts in the terminals of nerve fibres and that an intravenous injection of DOPA somehow through the formation of noradrenaline, profoundly influences the reflex transmission to neurones in the spinal cord, suggest that noradrenaline is a transmitter substance and some evidence from experiments with electrophoretical application of noradrenaline on interneurones in the spinal cord suggest that it has an inhibitory function (Engberg and Ryall 1966) Thus if the above interpretation is correct it is possible to study the effects of an activation of the descending 'noradrenergic system in isolation from all other supraspinal control systems by injecting DOPA intravenously in the spinal cat

The present investigation is concerned with the influence of this "noradrenergic" system on γ motoneurones as revealed by an injection of DOPA Experiments with some noradrenergic blocking substances (phenoxybenzamine, chlorpromazine) have suggested that noradrenergic pathways might be engaged in the control of γ motoneurones (Henatsch and Ingvar 1956 Ellaway and Pascoe 1965, 1968)

Muscle spindles are influenced by two types of γ motoneurones controlling the dynamic sensitivity and the static firing respectively of afferents with primary endings (Jansen and Matthews 1962 Matthews 1962) Only γ motoneurones of the dynamic type appear to be spontaneously active in the spinal cat (Alnaes Jansen and Rudjord 1965 Bergmans and Grillner 1968), while static γ motoneurones acquire a resting discharge after an injection of DOPA (Grillner Hongo and Lundberg 1967) These results were obtained with the indirect method of recording from spindle afferents with primary or secondary endings Since the results suggested differences in reflex behaviour of static and dynamic γ motoneurones it was desirable to record the activity in single γ motoneurones in order to find if they with respect to spontaneous activity and reflex activation fall in two categories This investigation is concerned with the activity in single γ efferents to flexors The reflex effects obtained from a variety of stimuli have been studied before and after an injection of DOPA Some of the results have been discussed in a preliminary report (Bergmans and Grillner 1967)

Methods

1 *Preparation*

Experiments were performed on 16 cats operated under ether anesthesia which was followed by anemic decortication (Voorhoeve 1960 as modified by Anden *et al* 1966a)

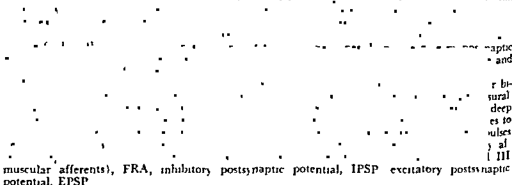
A laminectomy was performed from L3 to L7 leaving dorsal and ventral roots intact and a smaller one at lower thoracic level for spinalization Ipsi and contralateral cutaneous and muscular nerves were dissected and mounted for stimulation (all nerves dissected are listed under Abbreviations the tibial (Tib) and the peroneal nerves were often left intact) The femoral nerve and the nerve to gracilis were cut on the ipsilateral side The spinal cord and the hindlimb nerves were covered with liquid paraffin After the operation the cats were immobilized with gallamine triethiodide (Flaxedil May and Baker Ltd) and artificially respirated with a mixture of air and $O_2 + 5\ \% \ CO_2$ Blood pressure was continuously recorded A mixture of high and low molecular dextran was administered if the blood pressure dropped below 90 mm Hg The rectal temperature and the temperature of the paraffin pools was maintained between 36 5° and 38 5° C

2 *Stimulation and recording*

The peripheral nerves were stimulated with a condenser discharge (half decay 45 μsec) Single stimuli, short trains (intervals usually 2 5 or 3 3 msec) or a continuous train of pulses at variable intervals could be delivered to the peripheral nerves Single stimuli or short trains of pulses were never repeated more often than once every 3rd second Activity in the γ-efferents was recorded bipolarly with Ag—AgCl electrodes Afferent volleys were monitored through a surface electrode at the dorsal root entry zone of the appropriate segment

3 *Isolation and recording of single γ-efferents*

The activity of γ motoneurones was recorded in peripheral nerve filaments to flexor muscles mostly in branches of the nerve to the tenuissimus muscle The filament used for recording was taken as thin as possible The intact ventral roots were stimulated bipolarly through electrodes insulated but for the tip, in order to investigate the contribution of α and γ-efferents from the respective ventral roots One ventral root containing few γ-efferents was submitted to a progressive cutting with watchmaker forceps distally to the stimulation point, under continuous monitoring of the number of γ-efferents still in connexion with the spinal cord After the isolation of one or two γ-efferents from one particular ventral root, the other ventral roots were cut



muscular afferents), FRA, inhibitory postsynaptic potential, IPSP excitatory postsynaptic potential, EPSP

Results

1 *Spontaneous activity*

Fig 1 shows the activity in two γ efferents obtained in different preparations Stimulation of the intact ventral root in A and D respectively evokes a small and late spike at a fixed latency in an all or none fashion indicating that it represents a single action potential that is conducted at γ-velocity (*cf* Methods) The γ fibre in the left column (A—C) has a resting activity of 69 pps (B) that is abolished after an intravenous injection of DOPA (C) The fibre in the right column (D—F) on the other hand has a very low resting activity, 1 pps (E) that is enhanced (35 pps) after DOPA (F) Activity in these two filaments is thus influenced in a reciprocal manner by the injection of DOPA In order to establish that they represent different types of γ motoneurones and not only different reactions to DOPA due to differences of experimental conditions it was advantageous to record both types of neurones simultaneously Fig 2 shows the activity in a filament with two γ-efferents Before DOPA (B) the large diphasic unit is spontaneously active (63 pps) whereas the smaller but faster unit has no resting activity During an intravenous injection of DOPA a resting discharge appears in the small unit while that of the large one is decreased (C) Soon after the injection the small unit is dominating (D) and some minutes later the resting activity in the large unit has vanished but instead the small previously silent unit has acquired a high level of resting activity (25 pps) (E)

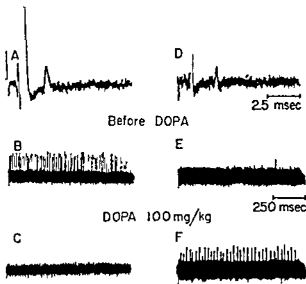


Fig 1 The influence of an intravenous injection of DOPA on the spontaneous activity in two γ efferents to tenuissimus recorded in two different experiments A and D show the activation of the γ-efferents by stimulation of the ventral root of S1 that had been subjected to successive lesions until only one γ efferent was still connected to the spinal cord L6 and L7 ventral roots were cut The unit in the left column (A—C) shows a spontaneous activity that is abolished after an injection of DOPA (100 mg/kg) The right series (D—F) illustrates a unit accelerated after DOPA Time calibrations as indicated in this and consecutive figures

Thus two types of γ motoneurones can be distinguished in the same preparation under the same conditions The present material consists of 15 neurones with no or very low resting activity in the spinal state which accelerated after DOPA and 7 units with a resting activity above 20 pps, which decelerated or were completely silenced after DOPA The latter type will be referred to as type A (*cf* Fig 1 A—C) and the former as type B (*cf* Fig 1 D—F) All neurones recorded from belonged to either type

Hunt (1951) claimed that the resting activity in the spinal unanesthetized cat was abolished after section of the dorsal roots Later investigations (Voorhoeve 1960 Lindsley and Eldred 1960) showed that there is a residual resting activity but nevertheless the results support the notion that the resting activity in γ motoneurones is maintained largely by the dorsal root afferent inflow These results refer only to the type of γ efferents that is spontaneously active in the spinal animal by Hunt and Paintal (1958) estimated to 40 % of the total population of γ motoneurones After DOPA spontaneous activity occurs in the type of efferent that previously was silent (B type) In order to see if this activity is maintained in a similar way the dorsal roots were cut in two experiments when B units exhibited resting activity The B unit recorded in Fig 1 decreased its resting activity from 35 to 30 pps when part of the S1 dorsal root was transected A further decrease to 16 pps was encountered on cutting the ipsilateral dorsal roots from L4 to S3 and after transection also of the contralateral roots the discharge frequency was 11 pps The marked decrease in discharge rate allows the conclusion that the resting activity that occurs after DOPA in B units at least partly depends on the dorsal root inflow

To give a more detailed account for the spontaneous activity, and in particular to see whether there is any difference between A and B units, a further a

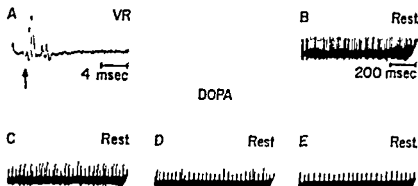


Fig 2 Reciprocal influence of DOPA on two γ efferents recorded in the same filament Stimulation of the L6 ventral root activates two γ efferents one small and one slightly larger diphasic unit (A) L7 and SI ventral roots are cut The latter unit is spontaneously active before DOPA (B), but decreases its activity during an injection of DOPA (C) Some minutes after the injection was completed this unit is silent (E) in contrast to the small one that has now acquired a spontaneous activity

regularity of firing and the distribution of interspike intervals was undertaken In Fig 2, the A unit (B) apparently has a higher resting activity than the B unit (E), but the latter, on the other hand, appears to have a more regular firing pattern The mean interval between consecutive spikes for the A unit is 14 3 msec (SD 4 6) and for the B unit 37 6 msec (SD 5 2) The relative variation in regularity of firing is conveniently expressed as the coefficient of variation (CV), *i e* the ratio between the standard deviation and the mean interval For the larger unit in Fig 2 spontaneously active before DOPA the CV is 0 32 and for the smaller (B type) active after DOPA 0 14 Thus there is a considerable difference in regularity of firing between these two units recorded in the same preparation Precautions were taken to control that the spontaneous activity was at a stationary level during the measurements The mean CV for 5 A units was 0 23 and for 7 B units 0 18 The coefficients for the different units are plotted versus the mean frequency in Fig 3 Each value is computed from the determination of at least 50 intervals The A units have a shorter mean interval than the B units Although there is a considerable overlap between the coefficients of variation of A and B units there might be a tendency for the B units to have a lower value *i e* a more regular firing pattern

The interval histograms in Fig 4 represents one A unit and one B unit The left histogram (A unit) has a positive degree of skew but the right appears to be more symmetrical For a comparison of the symmetry of different histograms a coefficient of skew can be estimated *i e* coefficient of skew $= 3\left(\frac{\text{mean interval median interval}}{\text{standard deviation}}\right)$

The mean value for the interval histograms of 5 A units was +0 43 (median +0 46) and for 6 B units +0 13 (median +0 15) The degree of skew for the different units can be compared in Fig 4 All the interval histograms of A units were positively skewed while the histograms of B units appeared to be more symmetric

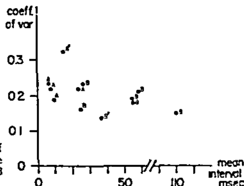


Fig 3 The relation between the coefficient of variation and the mean of at least 50 interspike intervals for 5 A units before DOPA and 7 B units after DOPA

In the discharge pattern of dorsal spinocerebellar tract cells (Jansen, Nicolaysen and Rudjord 1966) there is a relation between the duration of one interspike interval and the preceding one in that a short interval is generally followed by a long The present investigation did not reveal such a relation in the discharge pattern of γ-motoneurones of either type

2 *Reflex effects to γ motoneurones evoked from primary afferents*

a) *Reflex effects with short central delay*

γ-motoneurones can be reflexly activated with a central latency of 2—3 msec (Hunt and Paintal 1958) There is, however, a considerable variability in reflex excitability of γ-motoneurones in the spinal animal (Hunt and Paintal 1958) Alnaes, Jansen and Rudjord (1965) suggested from indirect evidence that only dynamic γ motoneurones could be reflexly activated in the spinal animal On the other hand, Grillner *et al* (1967) found reflex activation of static γ-motoneurones after an injection of DOPA In the light of these new data it seemed necessary to reinvestigate the

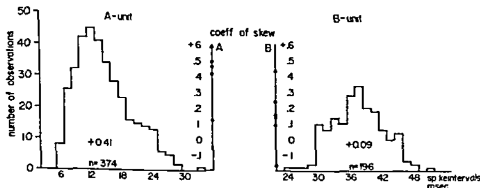


Fig 4 Histograms of the interval distribution of consecutive spikes in an A (n=374) and a B unit (n=196) The left histogram (A unit) has a positive degree of skewness (+0 41) but the right (B unit) appears to be more symmetric (+0 09) The coefficients of skew (*cf* text) are given for 5 A and 6 B units to the right and the left of the histograms respectively

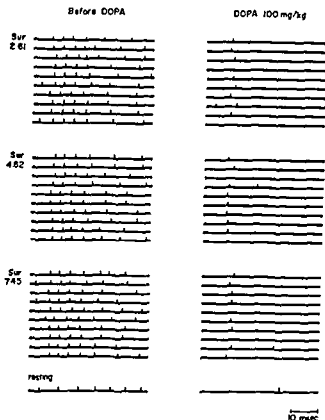


Fig 5 Schematic representa *of the reflex discharge of a* motoneurone of the A type in sponse to a single volley in sural nerve before and a DOPA Each horizontal line r resents one sweep and each v tical bar the occurrence of action potential The stimulat is set up in the beginning of ea sweep and the stimulation stren is given in multiples of the thre old for the most excitable fib (indicated to the left of ea series of 10 consecutive record The right series of records w obtained after an intravenous i jection of DOPA and the refl discharge is then smaller T lower two traces show the resti activity

matter with the direct method of recording the activity in γ efferents Since the shor latency reflex transmission from cutaneous group II and III muscular and joir afferents, *i e* the flexor reflex afferents (FRA) to α motoneurones is depressed afte an injection of DOPA it is of interest to establish whether a similar effect is exerte on the reflex transmission to γ-motoneurones The division of γ motoneurones into A and B units employed in the preceding section will be used also here

Fig 5 is a schematic illustration of the reflex activation of a type A unit Eacl line indicates one sweep (time) and each vertical bar shows the occurrence of one action potential The sural nerve is stimulated at the beginning of each sweep Each group of records represent 10 consecutive sweeps and already at a stimulus strength of 2 61 times threshold 4 spikes are linked to the stimulation The very early spikes are spontaneously occurring and it can be seen that they delay the reflexly elicited spike somewhat At a higher stimulation strength the spikes occur with shorter inter spike intervals In the right column the reflex responses after an injection of DOPA are shown Only one spike sometimes none occurs as a response to the same stimu lation as in the corresponding records in the left column Thus the reflex response from cutaneous afferents to γ-motoneurones of the A type is diminished after DOPA

Fig 6 is a similar schematic representation of the reflex activation of a γ-moto-

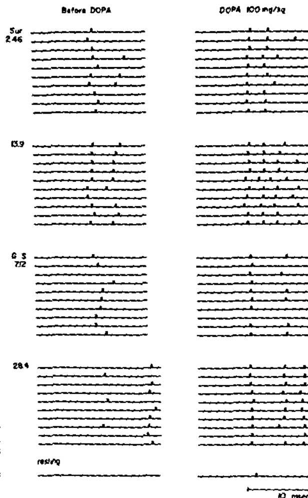


Fig 6 Reflex activation of a γ motoneurone of the B type before and after an injection of DOPA (100 mg/kg) from cutaneous (Sur) and muscular (G S) afferents at different stimulation strengths (single volleys). For details see Fig 5

neurone of the B type. Stimulation of the sural nerve at weak strength gives one spike sometimes two at a rather variable latency. After an i.v. injection of DOPA two sometimes three spikes occur at a shorter and more stable latency and with a shorter interspike interval. At higher strength this pattern is even more pronounced. Essentially the same type of reflex effects can be elicited from the nerve to gastrocnemius soleus (Fig 6 lower series). Thus in contrast to motoneurones of the A type the B type are more efficiently activated after an injection of DOPA. It is interesting that in response to the G-S stimulation at low strength the first spike occurs in 9 of 10 trials but at a higher strength of stimulation only in 3 of 10. The most likely explanation is that an early IPSP is evoked in addition to the EPSP.

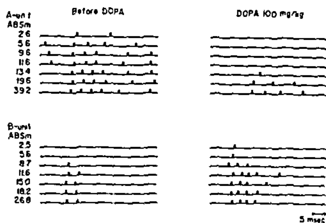


Fig 7 Reflex activation of an A and a B unit before and after an injection of DOPA (100 mg/kg) in response to single stimuli of increasing strength to a muscular nerve (ABSm) For details see Fig 5

The effect of increasing stimulation strength, given in multiples of threshold i shown in the same schematic way for an A and a B unit in Fig 7 The qualitativ effect of increasing stimulation strength is the same before and after DOPA bu the threshold for evoking a reflex discharge is lower before DOPA for the A unit anc after DOPA for the B unit Correspondingly the reflex activation is more effective a higher stimulation strengths

Another way of expressing the efficiency of reflex activation is to give the firing index, that is the ratio in per cent between the actual number of reflex responses and the number of trials (Hunt and Paintal 1958) Table I gives the firing indices for a B unit, which was activated in 5 % of the trials only at the highest stimulus strength from the G S nerve and somewhat more effectively from the sural nerve After DOPA the firing index is in all cases above 0 and even at a strength that yielded no

TABLE I

Before DOPA Firing index (%)		DOPA 100 mg/kg Firing index (%)					
Aff × thr	No of spikes 1	Aff × thr	No of spikes 1	2	3	4	5
G S 2 20	0	G S 2 70	75	0	0	0	0
6 80	0	5 30	94	0	0	0	0
10 0	0	10 0	100	15	0	0	0
40 0	5	40 0	100	100	100	72	11
Sur 1 34	0	Sur 1 47	100	35	0	0	0
2 10	33	2 10	100	40	7	0	0
11 2	76	11 2	100	92	78	14	0

spike before DOPA the firing index was 100 % after DOPA After DOPA not only one but several spikes occur in the reflex response At the highest stimulation strength (G-S) 4 spikes occur in 72 % of the cases as compared to one spike in 5 % of the cases before DOPA

Hence it can be concluded that the reflex activation of A units is less efficient (cf Fig 5, 7) after an intravenous injection of DOPA, while, on the other hand, B units are more excitable (cf Fig 6, 7, Table I)

The shortest latency of the reflex response evoked from the G-S nerve in Fig 6 is 8 7 msec The conduction time from the intact VR to the recording site is 3 0 msec and the central latency including the conduction time to the ventral root is minimal ly 2 0 msec (*cf* Hunt and Paintal 1958, Grillner *et al* 1969) Since the conduction distance is known, the conduction velocity of the afferent fibres could be calculated to be 46 m/sec, thus well within the group II range Reflex responses in A and B units were often elicited at a strength below 8 times threshold of the nerve (*cf* Fig 7) This strength can be assumed to be activating only group I and II afferents (Eccles and Lundberg 1959) In confirmation of Hunt and Paintal (1958), Eccles *et al* (1960) and Voorhoeve and vanKanten (1962), we found no evidence of excitatory actions from group I afferents while it can be concluded that group II afferents can exert an excitatory effect on flexor γ motoneurones of both types, as was stated by Voorhoeve and vanKanten (1962) This does not necessarily imply that these effects are elicited from spindle afferents with secondary endings, since it has been claimed that also other fibres conducting at group II range originate from muscle tissue (Barker 1967) It is of interest however, that group II afferents originating from muscle spindles have been shown to have an excitatory effect on α motoneurones to flexors thus conforming to the flexor reflex pattern (Laporte and Bessou 1959 *cf* Eccles and Lundberg 1959)

In this unanaesthetized preparation the flexor γ-motoneurones investigated (*i e* to tenuissimus and a few to semitendinosus) were almost invariably excited from ipsilateral cutaneous and muscular nerves (*cf*, however, Fig 6), but there was a considerable difference in excitability in confirmation of Hunt and Paintal (1958) A' units were usually activated with a train of spikes in response even to rather weak single stimuli B units, *i e* units with no or a very low resting activity, were sometimes poorly excited (*cf* Table I), but in other cases they responded with a train of spikes to single stimuli All γ motoneurones investigated could be activated if strong enough stimuli were used It is our impression that stimulation of cutaneous afferents gives a stronger reflex response Thus the reflex effects from ipsilateral nerves conform to the flexor reflex pattern There was no evidence for excitatory effects from group I afferents The effects from contralateral nerves were not tested in all neurones but when tested no consistent pattern was found Inhibition as judged from the depression of the spontaneous activity or mixed effects consisting of one or two spikes linked to the stimulation followed by a depression of the resting discharge rate or no effects at all were encountered

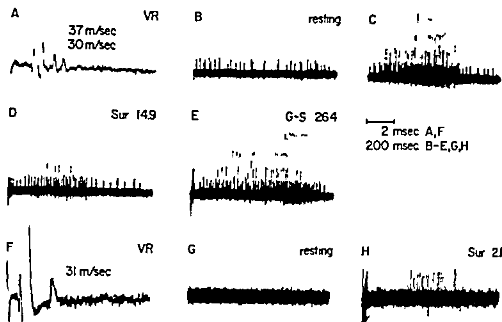
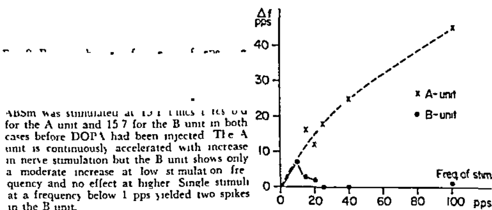


Fig 8 *The late reflex discharge in* γ *motoneurones of A and B type* occurring after an intravenous injection of DOPA (100 mg/kg) A—E are recorded from a filament containing two B units identified in A from the intact ventral root (SI) and restingly active after the injection of DOPA (B) L7 and I 6 ventral roots are cut Both α and γ motoneurones are activated with a central latency exceeding 100 msec by a short train (4 shocks) of pulses given to the sural or the G S nerve (D E) Stimulation strength is given in multiples of the threshold for the most excitable fibres Note that spikes with small spike size are recruited before those with larger spike size in the late discharge and also in the spontaneous bursts of activity occurring late after DOPA (C) Records F—H are from an A unit identified in F (SI ventral root) and with no resting activity after DOPA (G) A short train of pulses to the sural nerve yields a discharge with a long central latency (H

b) *Reflex effects with long central delay*

The short latency reflex transmission from FRA to α motoneurones is inhibited after DOPA but instead a longlasting reflex response (100—500 msec) occurs in both α and γ-motoneurones with a latency of 100—200 msec in the spinal animal (Anden *et al* 1966 a Static motoneurones to flexors do participate in this late discharge (Grillner *et al* 1967) but it is not known whether dynamic motoneurones participate

Fig 8 (A—E) illustrates two B units spontaneously active (B) after DOPA Stimulation of the G S and the sural (C D) nerves evokes a reflex response with a latency of 100 msec and with a duration exceeding 500 msec Also 3 α motoneurones in the filament are activated and it is interesting that the onset of reflex response is earlier in than in α motoneurones Within the latter group fibres with small spikes are activated before those with a large The order is reversed when the reflex response ceases (E) Also in the spontaneous bursts of activity that often occurs late after the injection of DOPA (C the neurones are recruited in the same order When record



ABSm was stimulated at 1 5 I times i ies o u for the A unit and 15 7 for the B unit in both cases before DOPA had been injected The A unit is continuously accelerated with increase in nerve stimulation but the B unit shows only a moderate increase at low st mulat on fre quency and no effect at higher Single stimuli at a frequency below 1 pps yielded two spikes in the B unit.

ing from thin filaments it can be assumed that there is a relation between spike amplitude fibre diameter, conduction velocity (Gasser 1941) and motoneuronal cell size as judged from the input resistance (Kernell 1966) It thus appears as if, in the late discharge motoneurones with small cell bodies are recruited before those with a large which can be interpreted in terms of the hypothes s that with the same amount of transmitter release a larger synaptic potential is generated in a cell with a high input resistance, i e a small cell as compared to one with a low (Katz and Thesleff 1957 Henneman Somjen and Carpenter 1965 Burke 1968) In F—H the activity in a γ motoneurone of the A type is illustrated after an injection of DOPA There is no resting activity but on stimulation of the sural nerve a reflex discharge is evoked with a latency of 200 msec Since the late reflex discharge could be elicited almost invariably after DOPA in both types of motoneurones it can be concluded that γ motoneurones of both A and B type as well as α motoneurones to flexors participate in the late reflex discharge

c) *Reflex effects evoked on repetitive nerve stimulation*

In order to have a constant reflex activation when investigating the response of primary endings to ramp stretches Alnaes *et al* (1965) employed weak repetitive nerve stimulation at 40 pps Under these conditions they found evidence for activation of dynamic but not static motoneurones Since in our experiments all motoneurones could be reflexly activated by single stimuli it was of importance also to consider the effect of repetitive activation at different frequencies

In Fig 9 the change in frequency (Δf) of an A and a B unit is plotted versus the frequency of stimulation The A unit spontaneously active at 75 pps is continuously accelerated as the frequency increases The B unit active at 0—2 pps was fired in response to each stimulus at very low frequencies of stimulation but at higher the reflex response was inhibited At 40 pps (employed by Alnaes *et al* 1965) no acceleration of the efferent fibre was found When B units were activated with several spikes on single stimuli the reflex response at low stimulation freq

could be considerable but as the frequency increased the reflex response decreased always being back to the resting level at a frequency of 20—25 pps. Hence there is an important difference between A and B units *in their response to repetitive nerve stimulation*. Alnaes *et al.* (1965) would have activated only A units.

d) *Reflex effects evoked on adequate activation*

Hunt (1951) found γ-motoneurones to flexors *to be activated from ipsilateral* and inhibited from contralateral skin regions, thus conforming to the flexor reflex pattern. Hagbarth (1952) and Eldred and Hagbarth (1954) demonstrated that there is a more complex organization of the reflex effects that can be elicited from the skin to α- and γ-motoneurones (*cf.* also Engberg 1964). To test if all γ-motoneurones are activated by adequate stimuli is of particular interest in relation to Alnaes *et al.*'s (1965) findings that on weak repetitive stimulation of peripheral nerves there was evidence only *of an increased dynamic fusimotor activity*.

In the present experiments the skin of the paw was stimulated by pressing the paw firmly between the index finger and the thumb. Both A and B units could be activated from the ipsilateral side both before and after an intravenous injection of DOPA. When spontaneous activity occurred, *i.e.* before DOPA for A units and after for B units inhibitory effects could be demonstrated from the contralateral side. With this rather crude adequate stimulus no threshold difference between activation of A and B units was detected. *It can be concluded however that all γ-motoneurones* investigated responded as expected from the flexor reflex pattern on adequate activation of this particular region of the hindlimb. Hence there is a difference between adequate stimulation and repetitive nerve stimulation at high frequency, the latter being unable to activate B units (*cf.* preceding paragraph).

Discussion

a) *Classification of A and B units*

The identification of A and B units must be based on the deduced properties of dynamic and static γ-motoneurones which have been inferred from studies of the discharges in muscle spindle afferents. These studies show that dynamic but not static γ-motoneurones have resting activity in the spinal cat (Alnaes *et al.* 1965, Bergmans and Grillner 1968). After an injection of DOPA, on the other hand, the *resting activity in dynamic γ-motoneurones* is decreased (Bergmans and Grillner 1968), while the activity in static is increased (Grillner *et al.* 1967, Bergmans and Grillner 1968). Evidence for a difference in reflex activation of dynamic and static γ-motoneurones in spinal cats was obtained by Alnaes *et al.* (1965), who found that weak repetitive nerve stimuli activated only dynamic γ-motoneurones. Since A units have all the described properties of dynamic and B units those of static γ-motoneurones, it is concluded that A units are identical with dynamic γ-motoneurones and B units with static. The proportion of A to B units (7 : 15) correlates rather well to the known proportion of dynamic to static γ-motoneurones supplying the tenuissimus (1 : 3) (*Bessou and Laporte 1966*). A *and* B *units* will be referred to as dynamic and static below.

b) *Reflex effects to static and dynamic γ-motoneurones*

The late reflex discharge that occurs with long central delay is evoked in both static and dynamic γ motoneurones Since these late reflex effects occur in all types of motoneurones they might be conveyed by a common pathway This suggestion is supported by the finding that the participation of α and motoneurones appears to be linked to each other the α motoneurones being recruited after the γ motoneurones (*cf* page 11 Fig 8)

α motoneurones and γ motoneurones of the dynamic and the static type are discharged with short central delay by single volleys in the FRA The reciprocal influence by DOPA on the reflex discharge in dynamic and static γ motoneurones and the inhibitory effects exerted by repetitive nerve stimuli only on the latter type of neurones might indicate that there are separate pathways mediating short latency excitation to the two types of γ motoneurones and that these pathways can be separately controlled The depression of the short latency reflex after DOPA (Fig 5 7) occurs in parallel in the pathways to dynamic γ motoneurones and α motoneurones The depression in the latter pathway is not caused by a direct effect on the α motoneurones and from indirect evidence it has been suggested that the effect is exerted at the interneuronal level rather than on primary afferent terminals (Andén *et al* 1966 a) It has been tentatively suggested that there is a linkage between α and dynamic γ motoneurones from experiments with intracellular recording (Grillner *et al* 1969) which is supported by the present findings Hence it is possible that the depression of the pathway to dynamic γ motoneurones also occurs at an interneuronal level

The early reflex discharge in static γ motoneurones is enhanced after DOPA This was unexpected since transmission in all previously investigated short latency pathways from FRA is depressed after DOPA (Andén *et al* 1966 a)

An increased excitability after DOPA either in static γ motoneurones or in the reflex pathway to them would account for this finding From recordings of the discharge it is not possible to distinguish between these possibilities Another factor that might be of importance is the level of primary afferent depolarization (PAD) in the terminals of the FRA The PAD may be at a lower level after DOPA than before (Andén *et al* 1966 a) Hence the transmission from the FRA to the interneurones in the reflex path to α and γ motoneurones would be enhanced If the reflex arc of static neurones in contrast to the other pathways was not depressed the observed increase in excitability could be explained by the removal of PAD That this should be of such a paramount importance for the excitability of static motoneurones is however to us less probable and we favour the idea of an increased excitability in one of the neurones of the reflex path Since noradrenaline postulated to be released by DOPA (*cf* Introduction) probably is an inhibitory transmitter (Engberg and Ryall 1966) it is more likely that the encountered increase in excitability is due to disinhibition than to direct excitation of one or more of the neurones within the reflex arc

To summarize the short latency paths to dynamic and static motoneurones are

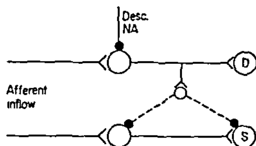


Fig 10 A model that could account for the reflex activation and the generation of spontaneous activity in dynamic (D) and static (S γ motoneurones and the mechanism of the reciprocal noradrenergic control One neurone can represent a chain of neurones Terminals with two branches are excitatory and those with filled circles are inhibitory The descending noradrenergic fibres are assumed to liberate an inhibitory transmitter

reciprocally influenced by an injection of DOPA and further the pathway to static γ motoneurones is inhibited by repetitive nerve stimulation in contrast to dynamic γ motoneurones (see Results page 15) A simple model that could account for these findings is presented in Fig 10 The short latency pathway to dynamic γ motoneurones (D) receives inhibition from descending noradrenergic fibres The effect is exerted at the interneuronal level in analogy with α motoneurones (see above) The pathway to static motoneurones (S) receives collateral inhibition from the pathway *to dynamic neurones No preferential location for this inhibition is evident* In the spinal state single volleys from ipsilateral peripheral nerves would give a reflex discharge in both static and dynamic motoneurones The pathway to static γ motoneurones would probably be less excitable due to the tonic afferent inflow causing inhibition of this reflex arc On repetitive nerve stimulation the first volley would discharge γ motoneurones of both types but the pathway to static would also receive inhibition through the inhibitory interconnexion With higher frequencies this inhibition would be effective also when the next volley arrives and prevent the reflex discharge An injection of DOPA would inhibit the reflex arc to dynamic γ motoneurones and consequently disinhibit the pathway to static neurones

It should be emphasized that other interpretations are possible It is of interest that some γ motoneurones to extensors receive recurrent inhibition (Ellaway 1968 *cf* also Brown Lawrence and Matthews 1968) but it is not known whether it is distributed to both types of motoneurones

c) How is the descending control of spontaneous activity in γ motoneurones exerted?

There is a relation between efficacy of transmission in the short latency reflex path and the spontaneous activity occurring in dynamic γ motoneurones before and in static after DOPA Results) *This resting activity is largely maintained* by the afferent inflow through the dorsal roots (*cf* page 4) Since the short latency path is active in the spinal animal the resting activity in dynamic γ motoneurones is probably maintained through this path Static γ motoneurones are spontaneously active when both the short and the long latency reflex pathways are open Since the late reflex pathway appears less discriminative (*cf* page 11) *we tend to favour* the possibility that the spontaneous activity in static γ motone maintained

through the short latency reflex path. We thus tentatively suggest that the spontaneous activity in dynamic and static γ motoneurones to flexors is maintained through the short latency reflex path. It is tempting to speculate how this control is exerted and to relate the disappearance of resting activity in one type of motoneurones to the occurrence to activity in the other type.

The model of Fig. 10 could account also for the reciprocal control of spontaneous activity in γ motoneurones. A continuous activity in the pathway to dynamic neurones would via collateral inhibition cause a depression of the pathway to static neurones but on inhibition of the former pathway the latter would be released. According to this model the spontaneous activity in the two types of γ motoneurones would have the same characteristics if the pathway to dynamic γ motoneurones was completely inhibited after DOPA and there were no other differences between static and dynamic γ motoneurones. In the present material the resting activity in static γ motoneurones tends to be lower than in dynamic and they also suggest some differences in the regularity of firing and in the symmetry of the interspike interval histograms. These differences are of considerable interest for further experiments but do not necessarily invalidate the model.

d) *Possible functional significance of the control of static and dynamic motoneurones*

α and γ motoneurones are coactivated in different reflexes (*cf.* Granit 1955) and also in the naturally occurring respiratory movements (Critchlow and Euler 1963, Euler 1966) and in hindlimb muscles during induced locomotion in the mesencephalic cat (Severin, Orlovskii and Shik 1967). Both dynamic and static γ motoneurones appear to be rhythmically activated in phase with the respiratory movements (Euler and Peretti 1966). Also in the flexor reflex that occurs with both short and long central delay do dynamic as well as static γ motoneurones participate (*cf.* Results). When considering the function of the stretch reflex loop the phasic activation of γ motoneurones has attracted most attention and particularly the hypothesis suggesting that the γ loop functions as an independent follow-up length servo for the initiation of movement (Merton 1953, Eldred, Granit and Merton 1953). No proof of this hypothesis has since been presented (*cf.* Hunt and Perl 1960, Matthews 1964, 1967) and experiments trying to estimate the gain of the stretch reflex loop have not lent support to the hypothesis (Granit 1958, Matthews 1959, 1966 b).

Although Hunt (1951) and Kobayashi, Oshima and Tasaki (1952) noted the occurrence of spontaneous discharge in γ motoneurones the implication of this has been given less attention. Provided that the interpretation of the effect of DOPA is correct the descending noradrenergic system controls the spontaneous activity in static and dynamic motoneurones to flexors in a reciprocal way. The noradrenergic fibres are unmyelinated (Dahlström and Fuxe 1965) and the conduction time from the brain stem probably exceeds 100 msec. Thus it is unlikely that the system is engaged in the control of fast movements with short duration but rather in modify

ing the background discharge and balancing between dynamic and static fusimotor activity This control is thus reciprocally organized in contrast to the coactivation of static and dynamic γ motoneurones in respiratory movements (Euler and Peretti 1966) and in the flexor reflex occurring with short and with long central latency (cf above)

However, the functional significance of the separation of γ motoneurones into a static and a dynamic type is by no means obvious It has been suggested (Jansen and Matthews 1962 Matthews 1964) that the function of dynamic γ motoneurones should be to provide a variable damping of the stretch reflex loop to suit any kind of movement Recent findings of Lennerstrand (1968) are relevant to this hypothesis He demonstrated that during simultaneous stimulation of static and dynamic γ motoneurones the response of a primary ending during a length decrement for example a muscular contraction is largely dependent on the activity in static fusimotor fibres independent of a concomitant dynamic γ activity On the other hand the afferent response during a length increment is largely dependent on the dynamic fusimotor activity This means that the primary ending is velocity sensitive to a length increment but not to a decrement when under simultaneous dynamic and static fusimotor bias It might be significant however that a decreased static activity means an increased sensitivity to velocity also during a length decrement

From these results it can be inferred that an activation of the descending noradrenergic system in the spinal cat decreases the dynamic response to a length increment but at the same time increases the afferent activity during a length decrement e g a muscular contraction Thus in this condition the muscle would oppose externally induced length increments less effectively but probably contract more efficiently since the afferent activity would not decrease abruptly during shortening (as under dynamic or without fusimotor activity) As pointed out above the descending noradrenergic system seems less well suited to participate in phasic shortlasting movements but eminent for setting the background level of static and dynamic fusimotor activity to suit a particular kind of movement On this background level of activity a phasic activation from either descending fibres or via spinal reflex arcs could be superimposed

No attention has here been paid to the secondary endings of the spindle which give an afferent signal linearly related to muscle length So far their only known reflex connexion to α (Laporte and Bessou 1959 cf Eccles and Lundberg 1959) and γ motoneurones (cf Results) are those of the flexor reflex (Sherrington 1910) It shall be noted however that group II afferents are accelerated after DOPA when at the same time the transmission from group II afferents tends to be favoured due to the removal of primary afferent depolarization (Grillner *et al* 1967)

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Properties of Clarke's Column Neurones

By

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Abstract

Eide E, L Fedina, J Jansen A Lundberg and L Vyklicky *Properties of Clarke's column neurones* Acta physiol scand 1969 77 125—144

Mechanisms responsible for the high efficacy of synaptic activation of the dorsal spinocerebellar tract (DSCT) was investigated with intracellular recording from Clarke's column cells in

The organization of synaptic connexions to the dorsal spinocerebellar tract (DSCT) has been extensively investigated (*cf* Lundberg 1964 Oscarsson 1965 and for more recent work Jansen and Rudjord 1965 Hongo and Okada 1967) Like many other sensory neurones DSCT cells are characterized by their very effective activation from primary afferents and the purpose of the present work has been to investigate the synaptic functions and cell properties responsible for the high efficacy of transmission to the DSCT Special attention has been given to the subgroup of DSCT neurones activated from large muscle spindle afferents (Ia) because these afferents have monosynaptic connexion also with motoneurones and a comparison with motoneurones with their well known properties expectedly would be valuable Furthermore a quantitative comparison between discharge rates in Ia DSCT neurones and

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Ia primary afferents is available as a basis for this work (Jansen, Nicolaysen and Rudjord 1966). The present report deals with properties and the activation of Clarke's column cells monosynaptically *activated from group I muscle afferents* henceforth referred to as DSCT cells (*cf.* however, Lundberg and Oscarsson 1960, Landgren and Silfvenius 1968). Unitary EPSPs and certain related observations are described in a separate paper (Eide *et al.* 1969). There is some overlap with the results of a parallel investigation by Kuno and Miyahara (1968). A preliminary report of some of our results has been given (Eide *et al.* 1967).

Methods

The experiments were made on spinal cats anesthetized with chloralose (60 mg/kg). The animals were operated under ether and chloralose was given after the end of the operation. Laminectomies were performed in the lower thoracic region and in the lumbar region (L2—L4). The dorsal part of the lateral funicle was dissected in the lower thoracic region and mounted for stimulation in order to allow antidromic identification of DSCT cells. The investigation is based on data from about 80 antidromically identified cells with a resting membrane potential above 40 mV. *For hindlimb nerves dissected see abbreviations.* The nerves to medial gastrocnemius, lateral gastrocnemius, soleus, plantaris, flexor hallucis and digitorum longus, extensor digitorum longus and tibial anterior were in intact connexion with the muscles.

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of 2 M potassium citrate or in some cases 3 M KCl. The principle diagram of the ca... ...ue follower and the circuit for current application has been given by Eide (1968). In all experiments the microelectrode was inserted through the dorsal column. Despite the usage of electrodes with broken tips (1—1.5μ) electrode blocking and "dimpling" of the cord due to sticking of the electrode to the white matter was common. This phenomenon was correlated with sluggish venous circulation and was less pronounced when the venous circulation was normal. The veins in the exposed lumbar segments traverse the dura separately from the roots and with a longitudinal opening of the dura the dura retracted and caused venous obstruction. In the later experiments *in this series we avoided making these extensive slits in the dura but opened only a small hole* in the region where the electrode was inserted. With this method electrode blocking and dimpling was less marked. This was important since our procedure of finding the cells involved systematic tracking as described by Hongo, Jankowska and Lundberg (1968.

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at if circulation is in good condition

This is an important guide in finding

focal potentials disappeared

PSP excitatory postsynaptic potential

AD antidromic PBSt posterior biceps-semitendinosus

...neus *longus* tertius and *brevis* *G S* *gastrocnemius*-

peroneal tibialis anterior and extensor digitorum

ndritic

Results

In accordance with the experience of previous investigators (Curtis *et al.* 1958, Eccles, Oscarsson and Willis 1961, Hongo and Okada 1967, Kuno and Miyahara 1968) the DSCT neurones are usually injured by the intracellular microelectrode. This is reflected in a high frequency injury discharge, decline of membrane potential and eventually inactivation of the spike mechanism. Such signs of injury were found in varying degrees in all the DSCT cells penetrated. Some cells however settled in an apparently steady state of moderate injury with membrane potentials of 50—60

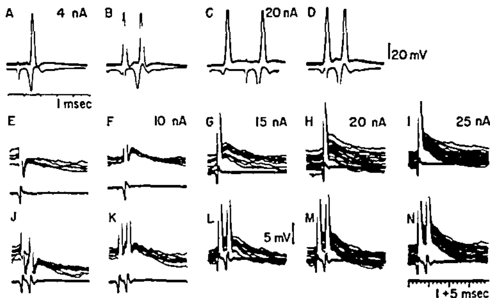


Fig 1 The effect of hyperpolarizing current on the action potential In this and most of the following figures upper traces are intracellular and lower traces from the dorsal column in the … ng 4 … K— … wing … ds in D for A—D and in L for E—N Superimposed traces in all records Resting membrane potential 62 mV K citrate electrode

mV With constant hyperpolarizing current the increased spike activity of these neurones could be controlled and the cell might remain apparently stable for prolonged periods (sometimes more than 2 hrs) generating action potentials of up to 90 mV Most of the observations that are presented below have been obtained from such penetrations and it is believed that they illustrate some of the physiological properties of the cell The possible distorsions introduced by cell injury and transmembrane polarizing currents which were usually employed should however be kept in mind in the interpreparation of the observations

The action potential

The spike potential is shortlasting and followed by a brief hyperpolarizing overswing (Fig 1) In 20 cells with spike potentials above 50 mV the duration measured to peak of the overswing ranged from 0 38 to 0 80 msec with a mean of 0 57 msec There is close agreement with the value reported by Kuno and Miyahara (1968) In cells in good condition the spike potential often shows only moderate signs of separation in IS and SD components In Fig 1 the two components are only barely visible even when a second antidromic spike is evoked after a short interval in a hyperpolarized cell (D) SD spikes can follow repetitive stimulation as high as 500/sec,

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A virtual absence of focal potentials in Clarke's column was reported by Curtis Eccles a Lundberg (1958) It has now been regularly found that if circulation is in good condi extracellular focal synaptic potentials can be recorded This is an important guide in find the Clarke's column When circulation deteriorated these focal potentials disappeared

Abbreviations DSCT dorsal spino cerebellar tract EPSP, excitatory postsynaptic poten IPSP inhibitory postsynaptic potential AD antidromic PBSt posterior biceps semitend nos SPM superficial peroneal muscles (peroneus longus tertius and brevis) G S gastrocnem soleus TA tibialis anterior DP deep peroneal (tibialis anterior and extensor digitoru longus) IS initial segment SD soma dendritic

Results

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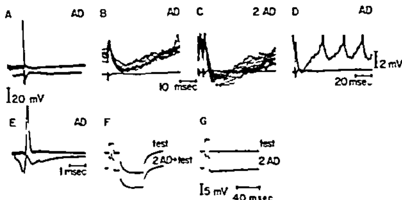


Fig 3 Summation of afterhyperpolarization and conductance change during the afterhyperpolarization Single antidromic volleys in A B D and E and double antidromic volleys in C F and G Observe summation of afterhyperpolarizations with double volleys in C and in the single trace in D where the antidromic spike is evoked soon after a spontaneous spike F and G show 50 averaged sweeps Hyperpolarizing pulses (7 nA) were applied through the recording electrode in F upper trace is the unconditioned test and lower trace the effect of two antidromic volleys In three similar measurements in this cell the increase in conductance was 5 9 and 13 % 10 nA steady hyperpolarizing current in A—E and 15 nA in F and G Traces in G as in F but without current pulse Resting membrane potential 55 mV K-citrate electrode

present After two antidromic spikes in motoneurones the delayed depolarization in response to the second is either decreased at very short intervals or considerably increased at somewhat longer intervals (Kernell 1964) In DSCT cells the delayed depolarization of the second spike reaches the same level of depolarization as that following the first spike and this is true even at different levels of polarization (Fig 1 J—N) Similar findings were made at longer intervals (Fig 1 C) only in a few occasions the second spike was followed by an increased delayed depolarization

To investigate the afterhyperpolarization it was necessary to stop the resting discharge with hyperpolarizing current In this state the hyperpolarization has a duration of 30—50 msec and its amplitude does not exceed 2 mV The afterhyperpolarization decreases markedly even with small hyperpolarizing current. The cell in Fig 2 had a resting membrane potential of 60 mV and discharged spontaneously in this state (A) as well as during passage of hyperpolarizing current of 4 nA (B With a current of 5 nA the spontaneous firing ceased (C) The striking decrease in afterhyperpolarization when the current was raised from 4 to 5 nA may be related to the disappearance of the resting discharge and with 15 nA there is no trace left A further increase to 25 nA did not give any reversal of the afterhyperpolarization The effective resistance of the cell was 1 05 MΩ which suggests that E and J were obtained at a membrane potential of 76 mV This is far below the equilibrium potential of 90 mV found for the afterhyperpolarization in motoneurones (Coombs Eccles and Fatt 1955 a It is unlikely that even the 25 nA current hyperpolarized the cell as far as to the potassium equilibrium potential

When two antidromic volleys were given in quick succession the amplitude of the

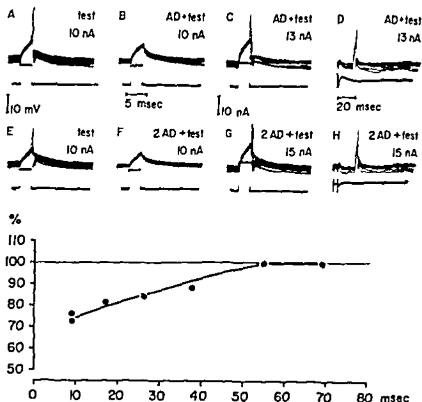
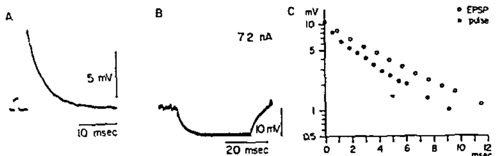


Fig 4 Excitability measurement during the afterhyperpolarization Depolarizing pulses wer given trough the recording electrode A Current pulse of 10 nA just fires the cell in al traces B No firing after single antidromic spik… 13 nA E—G As in upper records but with … and with the test firing the cell in about half … testing intervals at slower sweep In the gra… excitability (reciprocal of threshold) has been plotted as a function of time after a single anti dromic volley (ordinate Same cell as in Fig 3

afterhyperpolarization increased considerably (Fig 3 B and C) The hyperpolarization following either of the two shocks was of all or none nature if the strength of the second antidromic shock was chosen so that some of the stimuli failed to excite the axon two clear steps of afterhyperpolarization appeared It can therefore be concluded that descending inhibitory effects (cf Hongo and Okada 1967) did not contribute to the responses in B and C Record D in Fig 3 shows that in cells with a resting discharge there is also summation of afterhyperpolarization of an antidromically evoked and a spontaneously occurring spike The amplitude is increased and the interval to the next spontaneous spike prolonged

In motoneurones the conductance during the afterhyperpolarization increases by about 40 per cent (Ito and Oshima 1962) In DSCT cells there is a much smaller change as revealed by tests with the conventional rectangular pulse technique Record F illustrates an experiment with about 10 per cent increase in conductance (cf legend) However double antidromic volleys were employed for these measurements



[illegible] rane potential displacement evoked by hyper [illegible] I EPSP from PBSt B Membrane potential [illegible] gular current pulse A and B consist of 50 averaged sweeps C Semilogarithmic plot of the decay of the EPSP in A and of the establish ment of the hyperpolarization in B Resting membrane potential 49 mV 6 nA steady hyper polarizing current KCl electrode

since no appreciable change was observed during the afterhyperpolarization of a single antidromic action potential

The excitability during the afterhyperpolarization was investigated with brief de polarizing pulses applied throug the recording electrode (Fig 4) The intensity of the current was adjusted to threshold for firing During conditioning with a single anti-dromic volley the threshold current increases from 10 to 13 nA (A—D) and with double antidromic volleys from 10 to 15 nA The graph shows that the decreased excitability lasts for 50 msec which corresponds to the duration of the afterhyper polarization

The EPSP and the response to injected current pulses

Eccles *et al* (1961) reported that in many DSCT cells the monosynaptic EPSP from group I muscle afferents had a double decay the depolarization rapidly declined to a prolonged residium which was interpreted as caused by a prolonged transmitter ac tion In the present investigation this residual depolarization was not observed in hyperpolarized cells in good condition as is illustrated by the averaged records in Fig 5 The time course of the EPSP is very similar to that found in motoneurones (Eccles 1957) The semilogarithmic plot shows the approximately exponential decay with a time constant of 5 2 msec Record B shows the effect of an injected rectangu lar hyperpolarizing pulse The graph shows a rapid initial displacement of the poten tial which can be theoretically expected (Rall 1960) followed by an approximately exponential build up with a time constant of 4 0 msec i e rather similar to that found for the decay of the EPSP In two other cells the time constant of the EPSP decay was even shorter than that of the potential displacement produced by hyper polarizing pulses, 4 msec versus 5 4 msec for the pulse in one cell and 3 2 versus 6 2 msec in another cell These findings give no reason to believe that there is a long lasting transm tter action Likewise the short duration of unitary EPSPs (Eide *et al* 1969) suggests a shortlasting transmitter action at the synaptic sites on DSCT cells

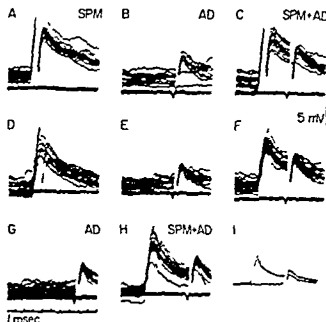


Fig 6 Interaction between the monosynaptic EPSP and the antidromic action potential A Monosynaptic EPSP was evoked from low threshold group I afferents of the DP and SPM nerves The SPM nerve was stimulated at a strength of 1 33 times threshold in A and C and at 1 26 times threshold in D F, H and I At the higher strength a spike is evoked in all traces from the rising phase of the EPSP at the weaker strength occasional spikes are evoked from the summit of the EPSP in D When the antidromic spike is superimposed on the EPSP in C F and H the delayed depolarization measured from the base line is larger than in the unconditioned records B E and G Observe that this also occurs with the long conditioning testing interval in H as is best shown in the averaged (20 sweeps) record I with superimposed traces of unconditioned and conditioned responses (same interval as in H) All records taken during passage of 15 nA hyperpolarizing current Voltage calibration is for the intracellular traces in A—H Spike potential without current 80 mV K-citrate electrode

We have confirmed that group I EPSPs may have a residual depolarization as described by Eccles *et al* (1961) The two phases of decay are often but not always found in cells severely damaged by the impalement Conceivably these EPSPs may be recorded from the soma the fast phase given by the damaged soma membrane and neighbouring region of the proximal dendrites and the slow phase by electrotonic spread from more distant intact regions of the dendrites However in some cases the late residual phase may have a slower decay than the group I EPSP in DSCT cells in good condition It is possible that the recording in these cases is from a dendrite and that the late phase represents the electronically slowed EPSP from other dendrites

Removal of the EPSP by the spike has been used to study the transmitter action in motoneurones (Eccles 1957) The interaction between EPSPs and spikes in DSCT cells gives results which at present cannot be unequivocally interpreted In A Fig 6 a spike is evoked from the summit of the EPSP and after the brief overswing a depolarization follows which is much larger than the delayed depolarization following the antidromic spike *In D the orthodromic stimulation was reduced to threshold for* firing Whether a spike is evoked or not from the summit of the EPSP the succeeding decay of depolarization follows approximately the same course and as in A The depolarization after the orthodromically evoked spike is larger than the delayed depolarization after the antidromic spike (E) The explanation for the prolonged depolarization in A and D could be either rebuilding of EPSP caused by survival of transmitter action or an increase of the delayed depolarization With regard to this problem it is relevant that a similar phenomenon occurs in the interaction between the EPSP and the antidromic spike at larger conditioning testing intervals (F H) In all these

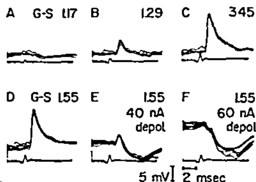


Fig 7 The effect of depolarizing current on the group I EPSP Graded stimulation of the G S nerve in A—C A disynaptic IPSP is evoked in A from low threshold group I afferents (probably Ia) and a monosynaptic EPSP from more high threshold group I afferents (probably Ib) E and F are sample records from the serie shown in the graph Observe reversal of the EPSP in F and in E and F the expected large increase in the IPSP with an onset soon after the summit of the EPSP Resting membrane potential 48 mV before and 30 mV after passage of the depolarizing current K-citrate electrode

records but to a decreasing degree with increasing intervals the depolarization following the conditioned antidromic spike is larger than the delayed depolarization of the unconditioned spike For a detailed comparison superimposed averaged records are shown for the longest interval (I) From what has been reported above in this section it is unlikely that EPSP rebuilding due to prolonged transmitter action could occur after long conditioning testing intervals in F and H It is tentatively suggested that the synaptic depolarization of dendrites causes a more effective antidromic invasion of them and consequently an increase in the delayed depolarization We have considered the alternative explanation that the dendritic region where the excitatory synapses are located is not invaded by the spike However since the group I afferents terminate on proximal dendrites (Szentagothai and Albert 1955) this explanation is hardly compatible with the hypothesis that the delayed depolarization recorded in the soma is caused by electrotonic spread of the dendritic spike to the soma

In the interpretation of the present results it must be considered that the EPSPs were recorded during passage of steady hyperpolarizing current The effect of current passage on the EPSP was investigated as illustrated in Fig 7 and 8 In Fig 7 a disynaptic IPSP was evoked by low threshold afferents presumably Ia) and a monosynaptic EPSP from higher threshold afferents (presumably Ib) The graph and the

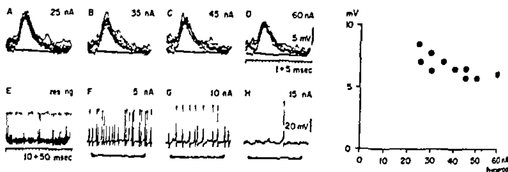


Fig 8 The effect on the group I EPSP from G S of steady hyperpolarizing current of intensities indicated in each record (A—D) and on the abscissa of the graph E—H Resting discharge and discharge during passage of steady hyperpolarizing current of indicated strengths Resting membrane potential before series in A—D 55 mV and afterwards when E—H were taken 50 mV KCl electrode

sample records (D—F) show the effect of depolarizing current The IPSP did not interfer with these measurements since its onset occurred after the peak of the EPSP With increasing depolarization the EPSP decreased and eventually reversed (F and graph) as has been found in motoneurones (*cf* Eccles 1957) In depolarized cell the EPSP increased with currents giving a repolarization towards the resting membrane potential The effect of a hyperpolarization on the EPSP is illustrated in Fig 8 The lowest value of hyperpolarizing current at which the test EPSP did not evoke a spike was 25 nA The current effect on the resting discharge (E—G) suggests that a hyperpolarizing current of 15 nA brought the membrane potential close to its resting value Hence in this experiment we do not take into account the effect of hyperpolarizing current in the range 10 nA above the resting membrane potential corresponding to some 10 mV potential change Hyperpolarizing currents above this value produce a moderate decrease in the amplitude of the EPSP This corresponds to the findings in motoneurones (Eccles 1957) This effect may well be caused by anomalous retification as shown for motoneurones (Nelson and Frank 1967)

It is important to employ hyperpolarizing current in the study of EPSPs in DSCT cells At a low level of membrane potential the orthodromic volley evokes a spike like potential which can be graded (Fig 3 in Curtis *et al* 1958) In these cases it is not *possible to differentiate between the EPSP and the local response or partial* spike evoked by it The largest group I EPSP observed during hyperpolarization to a level where the maximal EPSP did not evoke spikes was 30 mV Values between 20 and 30 mV were observed in a number of DSCT cells (*cf* also Eide *et al* 1969) The values are appreciably higher than those (about 10 mV) found in motoneurones in anesthetized cats (*cf* Eccles 1957) This difference between DSCT cells and motoneurones is not only due to a higher input resistance in the former The effective resistance measured in 10 good DSCT cells ranged from 0 9 to 2 3 MΩ with a mean of 1 4 MΩ These values are comparable with those found in large motoneurones (*Kernell 1966) However larger values for the input resistance of* DSCT cells have been reported by Kuno and Miyahara (1968)

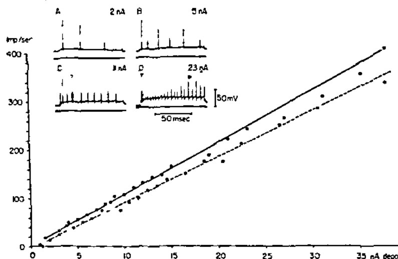


Fig 9 Firing produced by injected current pulse A—D Depolarizing pulses with current intensities given in each record These records are samples from a series used for the plot of the current frequency relationship In the graph open circles give the mean frequencies during the entire pulse and filled circles the mean frequencies when the first 30 msec of each pulse is excluded Resting membrane potential 55 mV 17 nA steady hyperpolarizing current K-citrate electrode

Firing by synaptic and injected currents

DSCT cells invariably respond to injected depolarizing current with a repetitive discharge which, except at current intensity very close to threshold, lasts as long as the current is applied Fig 9 shows the effect of a relatively short lasting pulse There is a linear relationship between current and frequency up to more than 300 imp/sec the slope is 10 6 imp/sec/nA if impulses are counted during the whole duration and 9 3 imp/sec/nA if the first 30 msec, the period of fastest adaptation is excluded In many cells the linear relationship was maintained for frequencies as high as 600/sec but at higher values the slope decreased Slope constants of the same order of magnitude were found when investigated with pulses of similar duration as in Fig 9 but occasionally higher values were encountered the maximal being 40 imp/sec/nA It is not known if DSCT cells with such steep slopes had a higher effective resistance

Fig 10 shows that full adaptation is only attained when the current is allowed to flow for more than 0 5 sec. The steady state slope constant for the cell in Fig 10 is 6 imp/sec/nA Measurements in the steady state as in Fig 10 in 7 cells gave values between 5 and 9 with a mean of 6 7 imp/sec/nA

The significance of the wide range and high degree of linearity of the firing frequency membrane current relationship is emphasized by the behaviour of the DSCT cells during simultaneous synaptic and injected current activation Our data from one cell are presented in Fig 11 The top records (A B) show that a hyperpolarizing current of 10 nA reduced the background firing from 120 to 40 imp/sec

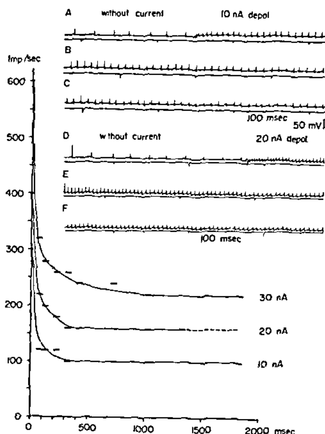


Fig 10 Firing by longlasting jected current A—F San records showing the initial per of current application 10 nA A—C and 20 nA in D—F A C and D, E F are continu records In the graph the h zontal bars show the peri during which impulses w counted Same cell as in Fig but no steady hyperpolarizing c rent

At both these levels of polarization a steady synaptic activation of the cell by a 10(load on the TA tendon increased the firing frequency by approximately 85 imp/s This is illustrated by the left hand lower graph of Fig 11 which shows the line summation of the effects of muscle stretch and the injected current

Further evidence of the linear behaviour of these cells is obtained by consideri the membrane potential changes to these two types of stimuli The depolarization the cell produced by the synaptic activity was measured when the cell firing w prevented by a 20 nA hyperpolarizing current The average depolarization of t cell in the steady state was approximately 10 mV (Fig 11 C) The displacement the membrane potential during current stimulation was estimated from the effecti resistance of the cell which was 0 9 MΩ This makes it possible to present the da on the firing frequency of the cell as a function of the estimated membrane potenti at the various levels of activation (Fig 11 right hand graph) The observatio appear all to fall along a straight line with a slope of 8 5 imp/sec/mV

The same type of data obtained from another cell is presented in Fig 12 Al *for this cell there was an algebraic summation of the effect of injected and synapt* current in the steady state Each nA of current added or subtracted 9 5 imp/sec a

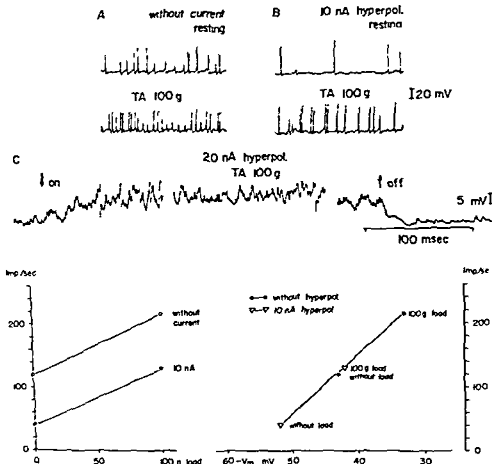


Fig 11 Comparison of the effect of injected current and muscle stretch Cell with monosynaptic group I EPSP from the DP nerve and effective activation by a light stretch of the anterior tibial muscle A Resting discharge and discharge by loading the TA tendon with 100 g (in the steady state about 0 5 sec after application of the load B Same but during 10 nA hyperpolarizing current C DC record of depolarization produced by loading the TA tendon with 100 g *during passage of 20 nA hyperpolarizing current Duration of muscle stretch* 0 75 sec left record onset of stretch with firing middle record after 0 35 sec and right record end of stretch The mean depolarization in the middle record is about 10 mV In the left graph the *impulse frequency is* plotted against the *load* without current and with 10 nA hyperpolarizing current The effective resistance of the cell was 0 9 MΩ and in the right graph the impulse frequency is plotted against the estimated membrane potential change produced by muscle stretch and injected current Resting membrane potential 43 mV K-citrate electrode

cording to the polarity of the current regardless of whether it was applied during or *without muscle stretch* and conversely a *100 g load on the muscle tendon increased* the firing rate by approximately 40 imp/sec irrespective of the intensity of simul*taneously injected current* The average steady state *depolarization of the cell caused* by the synaptic activation determined as in Fig 11 was approximately 6 mV *Examples of the displacement of the membrane potential during current pulses are*

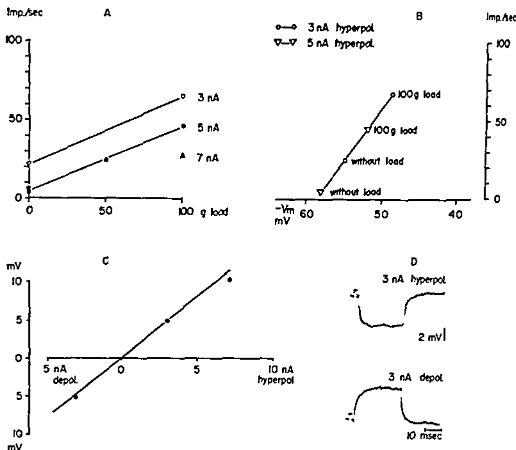


Fig 12 Comparison of the effect of injected current and muscle stretch A monosynap EPSP was evoked from low threshold group I afferents in the FDI nerve and a discha produced by light stretch of the FDI muscle A Impulse frequency without and with load (or 100 g) of the FDI tendon during hyperpolarizing current of 3 5 and 7 nA With 10 steady hyperpolarizing current a 100 g load gave a depolarization of 6 mV (not illustrated D Injected hyper and depolarization current pulses as indicated 25 averaged sweeps Steady state potential displacement by injected current pulses plotted from records such illustrated in D The effective resistance is about 1 6 MΩ B Impulse frequency plotted agai the estimated membrane potential displacement produced by muscle stretch and injected cu rent as indicated Resting membrane potential 50 mV KCl electrode

given in Fig 12 D and presented in the diagram C From this the *effective resistanc* of the cell was estimated to 1 6 MΩ Again the firing frequency of the cell presente as a function of the estimated membrane potential fits a straight line with a slope o 5 9 imp/sec/mV This type of relationship suggests an equivalence of synaptic anc injected current for the activation of the DSCT cells

We have some evidence that the above results are representative for the physio logical properties of the DSCT cells and not to any large extent influenced by the in jury caused by the impalement In one DSCT cell firing rates were compared before and after impalement This neurone was monosynaptically activated from group I

afferents in the G S nerve and effectively activated by light stretch of the soleus With extracellular recording the resting discharge was 10/sec and with a 100 g load on the soleus tendon the steady state firing rate was 53/sec After impalement of the cell (resting membrane potential 55 mV) the resting discharge was 22/sec and it increased to 63/sec with a 100 g load Hence under these different recording conditions a constant muscle stretch added virtually the same number of impulses 43 and 41 imp/sec respectively

Discussion

In this discussion of factors significant in explaining the effective synaptic excitation of DSCT the properties of these neurones will be compared with those of motoneurones (*cf* Eccles 1957)

There are important differences in the action potential of DSCT cells and motoneurones The shorter duration and pronounced hyperpolarizing overswing of the spike potential in the DSCT cells suggest that the permeability changes during the spike are adapted to allow high discharge frequencies Our results suggesting a high safety factor for impulse propagation from the initial segment to the soma are in complete agreement with those of previous investigators (Curtis *et al* 1958 Hongo and Okada 1967 Kuno and Miyahara 1968) *In addition it is postulated that propagation from the soma to the dendrites occurs with a higher safety in DSCT cells than in motoneurones* This postulate is based on the more regular occurrence of the delayed depolarization in DSCT cells than in motoneurones (Granit Kernell and Smith 1963 Kernell 1964 Nelson and Burke 1967) and its behaviour during double shock stimulation and polarizing current We have no independent evidence that the delayed depolarization is caused by the dendritic spike but base our interpretation on the reasonable suggestions forwarded by Granit Kernell and Smith (1963) and Kernell (1964) Support for this interpretation has been obtained from an analysis of a similar phenomenon in the stretch receptor neurone (Grampp 1966 b)

It is generally believed that the afterhyperpolarization regulates discharge frequencies in neurones (Eccles 1953) In view of the very high discharge frequencies in DSCT cells it was of particular interest to investigate their afterhyperpolarization mechanism There is general agreement that the afterhyperpolarization in DSCT cells is smaller and briefer than in motoneurones (Hongo and Okada 1967 Kuno and Miyahara 1968) After a single antidromic spike its magnitude usually does not exceed 2 mV and its duration is between 30 and 50 msec As in motoneurones (Ito and Oshima 1962) there is a summation of afterhyperpolarization from two antidromic spikes This summation may contribute to the negative serial correlation between the duration of neighbouring intervals in DSCT neurones activated by muscle stretch (Jansen *et al* 1966 but *cf* Walloe 1968) There is only a small conductance change during the afterhyperpolarization and it therefore seems doubtful whether the afterhyperpolarization as in motoneurones Coombs *et al* 1955 a) is caused by an increased permeability to potassium ions The alternative possibility that it may be caused by an active transport is unlikely in view of its high sensitivity to hyper

polarizing current (Fig. 3). Another possibility is that the membrane potential of these particular neurones to a larger extent than what has been generally assumed for nerve cells is dependent on the sodium conductance as, for example, in the pacemaker cells in the heart (Trautwein 1963). An inactivation of this sodium conductance by the spike and its slow build up possibly in conjunction with a decline of the potassium conductance could explain the afterhyperpolarization at low membrane potentials. Such a mechanism would have a high sensitivity to hyperpolarizing currents, and could operate without appreciable conductance changes during the afterhyperpolarization. This possibility is presented as a working hypothesis.

The discussion of the mechanism of the afterhyperpolarization also has relevance with regard to the resting discharge, which is so characteristic in DSCT cells (Laporte and Lundberg 1956, Jansen *et al.* 1966). A resting discharge remains after deafferentation and was assumed to be caused by an excitatory influence from interneurones (Holmqvist, Lundberg and Oscarsson 1956). The high degree of regularity of the resting discharge (Jansen *et al.* 1966) led to the suggestion that each interneuronal impulse only gives a small depolarization, i.e. that the presumed interneuronal bombardment should give a steady depolarization (Jansen *et al.* 1966). However an inherent pacemaker activity of the cells would equally well account for the regularity of the low frequency background firing.

While there seems to be important differences in the action potential in DSCT cells and motoneurones there is little evidence to suggest qualitative differences with respect to the EPSP evoked from Ia afferents. This is rather surprising in view of the previous findings regarding multiple discharges in DSCT neurones. Laporte *et al.* (1956) found that a pure Ia volley sometimes gave a double discharge and intracellular records revealed "rebuilding" of the monosynaptic group I EPSP after the orthodromic spike (Curtis *et al.* 1959, Eccles *et al.* 1961). This suggested a long lasting transmitter action and support for this hypothesis was obtained by Eccles *et al.* (1961) who showed two phases of decay of the EPSP, the second phase being very slow. The present investigation has revealed that as in motoneurones the group I EPSP in DSCT cells in good condition has a mono-exponential decay with a time constant that would be expected from the passive electrical properties of the membrane. In agreement with Kuno and Miyahara (1968) it is suggested that the transmitter action is shortlasting, probably not exceeding that in motoneurones. Several factors may contribute to the apparent rebuilding of the EPSP after the spike. First, the group Ia volley may be somewhat more asynchronous in its arrival to Clarke's column as the distance is longer than to motoneurones and the velocity is slowed down in the dorsal columns (Lloyd and McIntyre 1950). Since the spike potential of the DSCT cells appears very early on the rising phase of the EPSP and the spike has a very short duration, rebuilding of the EPSP may occur even though the duration of the transmitter action is not longer than in motoneurones (*cf.* also Kuno and Miyahara 1968). Second, the EPSP produced from the synaptic sites on proximal dendrites (Szentagothai and Albert 1955) may facilitate the invasion of the dendrites and give a larger delayed depolarization which would have the ap-

pearance of rebuilding (or survival) of the EPSP since in both cases the decay is determined by the passive properties of the soma-dendritic membrane. The delayed depolarization could give re-excitation as found in stretch receptor neurones (Eyzaguirre and Kuffler 1955, Grampp 1966 a). It would be of interest to study this mechanism in the cuneocerebellar tract, the forelimb homologue of DSCT. The neurones of this pathway often give 5—6 impulses in response to a single group I volley (Holmqvist, Oscarsson and Rosen 1962).

Expectedly Ia impulses would be more effective in firing DSCT cells than motoneurones since the maximal EPSP is larger, in our experiments 30 mV versus about 10 mV in motoneurones (Eccles 1957). Kuno and Miyahara (1968) report EPSPs up to 65 mV in DSCT cells but since they stimulated dorsal roots these large EPSPs may have been recorded from DSCT neurones activated from cutaneous afferents (*cf.* Lundberg and Oscarsson 1960).

DSCT cells differ from motoneurones (*cf.* Granit, Kernell and Shortess 1963) in that they invariably respond to injected depolarizing current with a maintained discharge. The measurement of the firing evoked by injected current shows a linear relationship between frequency and strength of current as has been found in motoneurones (Granit, Kernell and Shortess 1963) and in pyramidal cells in the pericruciate gyrus (Creutzfeldt, Lux and Nacimiento 1964). Kuno and Miyahara (1968) reported a non linear frequency-current relationship but this analysis is not comparable with ours since the frequency was calculated only from the interval between the first two spikes. In the steady state of firing after the initial adaptation the linearity holds true within a remarkably wide range in DSCT cells, while in motoneurones a secondary range with a steeper slope appears with stronger currents (Kernell 1965).

Granit, Kernell and Shortess (1963) described the frequency-current relation by the slope constant imp/sec/nA. However, for a meaningful comparison of membrane properties in different types of cells this constant should be expressed not in current but in depolarization produced by the current. Our mean value for the effective resistance gives a slope constant of about 4.8 imp/sec/mV. Kernell (1966) reports a slope constant of 2 imp/sec/mV for the primary range of firing in motoneurones. Also for cortical cells a slope constant of about 2 imp/sec/mV can be calculated from the data given by Creutzfeldt *et al.* (1964). Like motoneurones cortical cells have large and longlasting hyperpolarizations between 50 and 80 msec (Phillips 1956, Creutzfeldt *et al.* 1964). The higher slope constant in DSCT cells correlates with their smaller and briefer afterhyperpolarization.

In the secondary range of motoneuronal firing Kernell (1965) found a slope constant of 4.6 imp/sec/nA (as compared with 1.7 in the primary range). Using the value of 1 MΩ for the effective resistance in large cells (Kernell 1966) and assuming that the resistance is not radically changed in the secondary range it appears that this constant expressed in mV is of the same order as for DSCT cells. Possibly the afterhyperpolarizations in motoneurones in the secondary range of firing and in DSCT cells have a similar operative power.

Kernell (1965) has correlated the slope constant in the secondary range of firing with a changed time course of the afterhyperpolarization which seems to be associated with the disappearance of the delayed depolarization and possibly caused by a blockage of dendritic invasion (Granit, Kernell and Smith 1963 *cf* however Kernell 1968) The finding that the frequency current relationship is linear in DSCT cells within a much wider range than in motoneurones may be associated with the higher safety factor for impulse propagation between the different parts of the DSCT neurone (*cf* above)

The prediction (Granit Kernell and Shortess 1963) that experiments with current injection gives information, which is meaningful with regard to the synaptic activation of neurones is supported by the finding of a linear addition between the discharges evoked by injected current and muscle stretch An even more direct proof was obtained from the two cells in which the synaptic depolarization by muscle stretch was measured and compared with depolarization produced by injected current Since the depolarizations evoked by the synaptic current and the injected current have identical effect on the firing rate it is postulated that the extrinsic and intrinsic currents generate impulses at the same site, presumably the initial segment as has previously been suggested for the generation of impulses by single orthodromic volleys (Curtis *et al* 1958) The linear frequency current relationship within such a wide range is important in understanding the linear relationship between the mean frequency and muscle length found in Ia DSCT neurones (Jansen and Rudjord 1965) The finding that the slope constant (imp/sec/mm muscle length) is approximately the same in DSCT cells as in primary Ia afferents (Jansen and Rudjord 1965) must be conjointly determined by the intensity of the synaptic current evoked from all Ia afferents converging to each DSCT cell and the effective resistance of the cell which determines the depolarizing effect of the synaptic current

The properties of motoneurones have often been taken as characteristic for neurones in the CNS Our findings and those of Kuno and Miyahara (1968) show that DSCT cells in many respects have other properties which seem well adapted for their function to transfer sensory signals and operate over a wide frequency range Possibly this applies not only to other sensory neurones but also to other types of neurones in the CNS Hongo and Koike (to be published) have found that spinocervical tract neurones in many ways resemble DSCT neurones and to some extent this holds true also for spinal interneurones (Haapanen Kolmodin and Skoglund 1958 Hunt and Kuno 1959) The motoneuronal properties on the other hand seem well suited to subserve low frequency firing and a high degree of integration

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E... ...dendrite and axon excitation in

G... adapting stretch receptor

G... ...ns in the slowly adapting ... after depolarization *Acta*

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Unitary Components in the Activation of Clarke's Column Neurones

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Abstract

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In response to muscle stretch unitary EPSPs were recorded from group I activated dorsal spinocerebellar tract (DSCT) neurones The size of the different unitary EPSP varied appreciably the largest was about 5 mV The rise time and decay times of the unitary EPSPs varied only between 0 2—1 msec and 1—3 msec respectively The estimate of the number of primary

The probability of firing of a neurone through a presynaptic pathway is largely determined by the number of primary afferent fibres converging on to the cell and by the synaptic effect of each presynaptic impulse The latter can conveniently be measured as the unitary EPSP (u EPSP) for excitatory synapses Jansen Nicolaysen and Rudjord (1966) suggested that large u EPSPs might explain the characteristic irregularity of firing of dorsal spino-cerebellar tract (DSCT) neurones when adequately activated by muscle stretch and this suggestion has since been supported quantitatively by Walloe (1968) However the earlier experimental studies on the synaptic activation of the DSCT neurones contain no report of large u EPSPs (Curtis Eccles and Lundberg 1958 Eccles, Oscarsson and Willis 1961 Hongo and Okada 1967) In a paper which appeared during the preparation of this article Kuno and Miyahara (1968) have described u EPSPs of DSCT neurones some of which had large amplitudes Their origin in a particular group or groups of primary afferent fibres were not determined In the present examination of the question large u EPSPs have

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been demonstrated during adequate synaptic activation of the cells, and some of their properties shall be described Some of the results have been presented in a preliminary report (Eide *et al* 1967)

Methods

The present observations were obtained during the series of experiments reported in the preceding paper (Eide *et al* 1969)

Results

Unitary EPSPs Even without any specific synaptic input the majority of DSCT units showed appreciable, quite rapid fluctuation of membrane potential (Fig 2) When the appropriate muscle was stretched the amplitude of this synaptic noise increased and there was usually a shift of the DC level of the membrane potential in the depolarizing direction (Fig 12, Eide *et al* 1969) A precise analysis of the additional synaptic noise generated by the muscle stretch was impossible in units with pronounced resting synaptic noise However, in 9 of the 24 DSCT units examined the resting membrane potential was sufficiently stable to permit a clear identification of the components of the synaptic activity evoked by the stretch In these cases the synaptic activity consisted of characteristic rapid depolarizations followed by a slower more or less exponential return of the resting potential towards its resting value The character of this 'synaptic noise" can best be illustrated by some original records shown in Fig 1 Without load on the tendon there were only small and irregular fluctuations of the membrane potential (A) On loading the tendon the typical fluctuations appeared Their time course agrees in general with the time course of the EPSP elicited by a synchronous group I volley (Eccles *et al* 1961 Eide *et al* 1969) At low levels of stretch a single u EPSP might recur at regular intervals (Fig 1 C) The degree of regularity measured by the spread of the distribution of intervals between the u EPSPs was the same as that of the highly regular firing pattern of muscle stretch receptors (Eide *et al* 1967) At higher degrees of stretch the same u EPSP could be recognized from its time course and amplitude (Fig 1 D) Its rate of appearance however was higher and this agrees with an origin in a muscle stretch receptor The frequency increase was within the range of static sensitivities of the primary endings of soleus muscle spindles (Jansen and Matthews 1962) During prolonged extensions there was furthermore a slow progressive decline in the frequency of the u EPSP (Fig 1 B C) corresponding to the adaptation of the muscle stretch receptors (Matthews 1933) With increasing muscle stretch additional unitary, rhythmic EPSPs were recruited (Fig 1 D) When two or more u EPSPs coincided in time their depolarizations were summated

These observations establish these potential fluctuations as u EPSPs with a high degree of certainty In addition they provide some less direct information on the type of receptors activating the neurone under study The lowest threshold u EPSP was evoked for 1 5 sec with only a 10 g load on the tendon During the loading transient at least two or three other units were also present An even more pro-

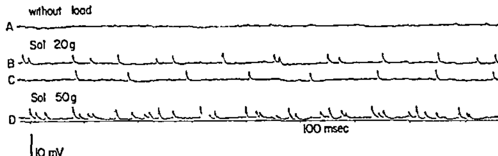


Fig 1 Unitary EPSPs of group I activated DSCT neurone A Stable resting membrane potential without load on the soleus tendon B 20 g load on soleus tendon about 4 sec after the loading transient C Same about 7 sec after loading D 50 g load on soleus tendon about 8 sec after loading transient Resting membrane potential 60 mV K-citrate electrode 10 nA hyperpolarizing current

nounced transient activation was observed with *20* and *50 g loads* This agrees with the high dynamic sensitivity of the primary endings of muscle spindles The tendon organs are less dynamically sensitive and their thresholds to passive extension of the soleus muscle is higher than that of the receptors generating the u EPSPs of Fig 1 This does not exclude that tendon organ afferents may converge on Clarke s column neurones activated from primary endings of muscle spindles such as suggested for instance by Eccles *et al* (1961), and we have no independent evidence on this somewhat controversial point

The main problem about attributing the u EPSPs of Fig 1 to activity in first order fibres from spindle primary endings is the absence of synaptic activity before the muscle was loaded It is common experience that something like 50 % of the primary endings of soleus fires spontaneously at a low rate even in the de efferented preparation With a fair number of afferents converging on the cell (see below) it is unlikely that they should all lack spontaneous activity The spontaneous activity is, however very sensitive to the experimental conditions and might well be less common in the present experiments with rather extensive liberation and separation of the individual components of the triceps muscle Neither can we exclude a possible effect of the anesthetic on the spontaneous activity of the primary endings This inference is supported by the observations of two other Clarke s column cells in the same experiment. These were activated by equally low threshold stretch receptors and showed only very small and uncharacteristic fluctuations of membrane potential in the absence of muscle stretch

One group I activated unit of the present series required approximately 50 g load on the TA tendon for stretch activation Presumably this was activated from tendon organs of the muscle In other respects the u EPSPs of this unit behaved like the others described below

As mentioned above u EPSPs might also be present before the muscle was stretched These u EPSPs might be similar to the stretch activated ones An example

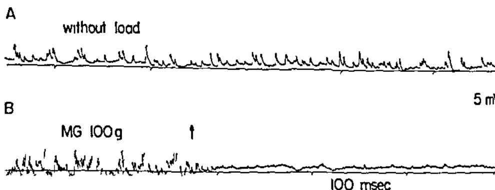


Fig 2 Unitary EPSPs of GM DSCT neurone A Muscle unloaded initially B 100 g load GM tendon release at arrow Resting membrane potential 40 mV K citrate electrode 20 n hyperpolarizing current

is given in Fig 2 The origin of these EPSPs have not been definitely determined In some instances such resting synaptic activity would disappear in the period immediately following release of the muscle from a rather heavy load (Fig 2) and recover gradually during the following few secs A similar behaviour is commonly found in muscle stretch receptors Hence it is suggested that this resting synaptic activity is due to primary afferent activity In other cells however the resting synaptic activity would continue without apparent alterations also in the release period Such activity did probably not originate in the muscle that was stretched It may be caused by interneuronal synaptic activity or by primary afferent activity from other sources

However the important point is the positive identification of some u EPSPs as generated by primary afferent activity The rise time of the u EPSPs was rather uniform as illustrated by the sample records (Fig 3 A) and in the scatter diagram of the rise time versus peak amplitude of u EPSPs recorded in the same cell (Fig 3 B) Some of the scatter is due to genuine differences in rise time of different individual EPSPs This is illustrated by Fig 3 C which shows a similar plot of the rise times and amplitudes of one individual EPSP recorded from the same cell The scatter is clearly less than in Fig 3 B and since errors of measurement and recording noise were the same for the two groups of data it appears that there were small but significant differences in rise time and rate of rise of individual u EPSPs This applies to all units of the present series The rise times of the u EPSPs did usually not vary by more than a factor of three In the one cell with the most pronounced differences in time course of the u EPSPs their rise times ranged for 0 2—1 msec

The time course of decay of the u EPSPs was also found to vary moderately In three different cells the time to 50 % decay ranged between 1 and 3 msec Data from the one cell in which 4 individual u EPSPs could be followed are given in the scatter diagram of Fig 4 It appears that there are considerable overlap between the decay times of the different units but the largest and most rapidly rising one appears to be the most rapidly decaying one as well

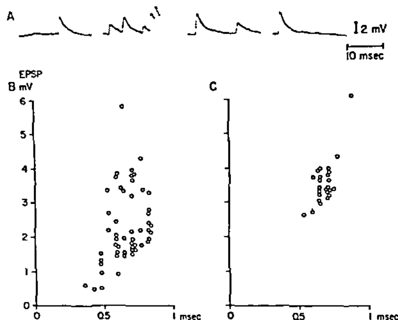


Fig 3 A Sample record of unitary EPSPs from the cell of Fig 1 Rapid time base B Rise times to peak of a series of consequtive unitary EPSPs of one neurone plotted against amplitude of EPSPs Same cell as A Data obtained early after loading transient with a 50 g load B Rise times versus amplitude plot of one individual unitary EPSP Same cell as A Data obtained from a period with 20 g load on soleus tendon

In conclusion it should be emphasized that the time course of the u EPSPs of Clarke s column neurones is rather uniform when compared with the corresponding Ia activated u EPSP of motoneurones These have been reported to have rise times between 0 25—2 5 msec and decay times (to $\frac{1}{e}$ x peak amplitude) between 1 and 10 msec (Burke 1967)

A further property of the DSCT neurones which prevented the determination of the time course and the amplitude of the u EPSPs was often observed At a certain

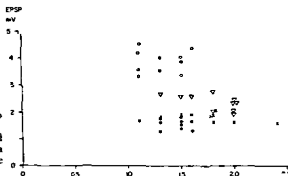
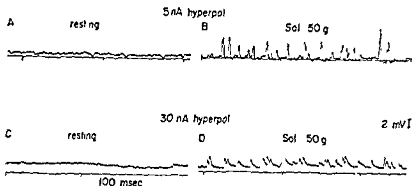


Fig 4 Decay times from peak to 50 per cent decay of four different unitary EPSPs plotted against peak amplitude The data from each unit has a separate symbol Same cell as Fig 1



degree of depolarization the cells tended to generate local responses or abortive sp in response to synaptic excitation This is illustrated in Fig 5 With a 5 nA hyp polarizing current the resting membrane potential of this cell was stable and mod ately noisy (Fig 5 A) During stretch activation (B) sharp fluctuations in membra potential occurred These were of variable amplitudes often with equally rap rising and falling phases With a 30 nA hyperpolarizing current the resting me brane potential was less noisy (C) and u EPSPs of typical time course appeared stretch activation of the cell (D) The contamination of the u EPSPs with local r sponses was sometimes difficult to recognize during the experiment Such recor were obviously useless for the analysis of the properties of the u EPSPs Previous published records of resting synaptic noise (Eccles *et al* 1961) may largely consist local responses

The EPSP of Clarke's column cells A possible alternative method to study the EPSPs and the number of afferent fibres converging onto each neurone is by electric stimulation of the muscle nerve Grading the electrical stimulus finely one may ob serve steps in the development of the summated EPSPs and these steps correspon to the recruitment of one additional afferent nerve fibre An example is shown Fig 6 It is a record of superimposed EPSP elicited by a constant stimulus strength

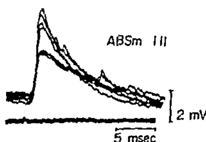


Fig 6 Compound EP ... nerve stimulated by ... Superimposed sweep ... mV K citrate elec

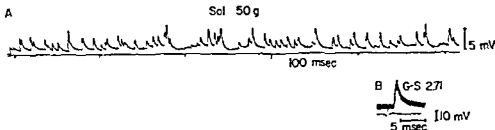


Fig 7 A. Unitary EPSPs of DSCT neurone during a 50 g load on the soleus tendon 10 nA hyperpolarizing current B Maximal group I EPSP to GS nerve stimulation. 60 nA hyperpolarizing current. Resting membrane potential 60 mV K-citrate electrode

Two sizes of EPSPs were evoked presumably because the highest threshold nerve fibre was just activated in about 50 % of the trials In some cells three or four such steps might be determined but in many other cells the steps were difficult to distinguish and the EPSP appeared to grow almost continuously with increasing stimulus intensity within the group I range This is presumably due to the fact that as more and more u EPSPs are recruited, each of them with an appreciable random variation in amplitude (Fig 1 and further description below) the total amplitude variation will increase, and possible steps due to the addition of new units will be obscured Accordingly, the method of finely grading the EPSPs did not appear suitable for the study of u EPSPs nor to determine the degree of convergence on the Clarke's column cells

The maximal group I EPSPs are useful by providing a measure of the total group I input to each cell This, as has been stated repeatedly before may be very powerful Group I EPSPs of 15—25 mV peak amplitude were frequently observed The ratio between this maximal group I EPSP and the average size of the u EPSPs should provide an estimate of the number of primary afferent fibres converging onto the cell The average size of the u EPSP was estimated from records like Fig 7 A with a strong stretch of the muscle, sufficient to activate all its primary endings These receptors usually have thresholds less than 20 g (Hunt 1954 Harvey and Matthews 1961) From such records the amplitudes of all u EPSPs in an 0 2 or 0 4 sec period were measured and their average size calculated During this period all the primary endings converging onto the cell should have generated at least some u EPSPs Except for the possibility of occasionally missing some of the smaller ones an acceptable estimate of the average unit size should be obtained The data from four different cells are given in Table I

The number of fibres converging onto each cell has been calculated as the ratio between the max EPSP and the average unit size multipled by the factor

1
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TABLE I

Unit	Muscle	Load	Max EPSP	Average unit size	Convergence
U^{18}	Sol	50 g	23 mV	2 75 mV	12
U^{14}	Sol	50 g	17 mV	1 6 mV	14
		100 g		1 8 mV	13
U^{10}	GM	20 g	14 mV	1 06 mV	16
U^{4}	FDL	100 g	6 mV	0 56 mV	12

This factor corrects for the nonlinear summation of the EPSP (Martin 1966), and 70 mV was used for V_0 (the driving potential of the EPSP) The data suggest that a limited number, 12—16 primary afferent Ia fibres converge onto each second order DSCT cell

The determination of the maximal EPSP is subject to some uncertainty To measure the maximal EPSP it was necessary to prevent the cell from firing This required a strong hyperpolarizing current The estimate of convergence given in Table I is obtained from measurements of the maximal EPSP in strongly hyperpolarized cells while the u EPSPs were measured at a membrane potential which presumably was only slightly above the resting level We have, however, in a limited number of cells, examined the effect of hyperpolarizing currents on submaximal EPSPs and our observations suggest that the Clarke's column cells (Eide *et al* 1969) in this respect behave rather like motoneurones Hyperpolarizing the membrane beyond the value of the resting membrane potential has little effect and may even moderately reduce the amplitude of the EPSU (Coombs, Eccles and Fatt 1955) We have therefore ignored this complication in the estimate of the degree of convergence

Firing threshold In addition to the total number of primary afferents converging onto each second order neurone and the size of the u EPSPs the firing threshold of the cell will influence the transfer of the signal and the firing pattern of the second order neurone It is however difficult to obtain an estimate of the normal firing threshold of the Clarke s column neurones This is because the cells were injured by the microelectrode and most of our useful records were obtained during the passage of a constant hyperpolarizing current (see Eide *et al* 1969) This current undoubtedly affects the size of the u EPSPs as well as the firing threshold of the cell and not necessarily to the same extent in terms of the membrane potential The current employed was usually the smallest which would prevent the cell firing without a load on its muscle, and this presumably means that the cell membrane potential was raised just above its normal resting level We therefore believe that our measurements of firing threshold and amplitude of u EPSPs are not too far from the physiological values The best measurements of firing threshold was obtained during stretch activation of sufficient intensity to fire the second order neurones (see Fig 5 D) For two of the three cells from which such observations

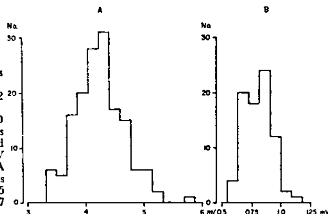


Fig 8 Distribution of amplitudes of individual unitary EPSs A Mean amplitude 4 3 mV, SD 0 42 mV Number of observations 151 B Mean amplitude 0 8 mV, SD 0 12 mV Number of observations 81 The data of A was obtained from a neurone with a 60 mV resting potential during 10 nA hyperpolarizing current B was obtained from a cell with a 35 mV resting potential during 17 nA hyperpolarizing current

were obtained, the average size of the u EPSPs was 2 75 and 1 3 mV and the firing threshold was 5 8 and 2 9 mV, respectively For the third cell the values were 0 95 mV (average EPSP) and 6 mV (threshold) It appears therefore that for these three cells the simultaneous occurrence of a presynaptic impulse in 2 1, 2 2 and 6 3 first order fibres on the average would be sufficient to activate the second order cell Of these figures there is reason to stress that as little as between 2 and 3 presynaptic impulses may fire the postsynaptic neurone

Quantal content of the unitary EPSP A final property of the u EPSP deserve consideration Even an individual u EPSP shows appreciable amplitude fluctuations (Fig 1) The most common explanation for this variation in amplitude, analyzed in great detail for the neuromuscular junction (del Castillo and Katz 1954) is that there is a quantal release of transmitter from the presynaptic terminals and that the release of each quantum is governed by a probability law If so the fluctuations of amplitude of the u EPSPs are due to variations in the number of quanta released by each presynaptic impulse For the neuromuscular endplate the Poisson distribution describes the amplitude fluctuations adequately (Martin 1955), and it appears reasonable to adopt the same model for the synaptic transmission to the Clarke s column neurones, at least initially

Examples of the distribution of amplitudes of individual u EPSPs one with a large and one with a small average amplitude are given in Fig 8 It appears that the distributions are fairly symmetrical and approximately gaussian This applies to the amplitude distributions of all the u EPSPs which could be confidently identified in the present material, and it indicates that the average number of quanta released must be fairly large, since the Poisson distribution approximates the normal distribution only for large average number of events This is supported by the observation that we have been able to follow a rhythmic u EPSP for many hundred occurrences without any failures in the train

TABLE II

Unit	u EPSP	No. of obs.	$\bar{x}$ mV	SD mV	$m = \frac{1}{(CV)^2}$	$q = \frac{\bar{x}}{m}$ mV
15	(1)	69	4.3	0.43	100	0.043
	(1)	82	4.3	0.38	117	0.037
	(2)	31	2.0	0.18	121	0.017
	(2)	64	2.5	0.23	110	0.023
	(3)	28	1.8	0.20	79	0.023
	(4)	24	1.4	0.23	36	0.039
10	(1)	23	1.7	0.18	79	0.021
	(1)	24	0.9	0.12	61	0.015
	(2)	31	1.5	0.31	25	0.060
12	(1)	28	1.2	0.21	35	0.034
	(2)	32	1.3	0.17	58	0.022
	(3)	24	1.8	0.22	65	0.028
5	(1)	56	0.95	0.15	39	0.024
4	(1)	81	0.75	0.12	42	0.018
1	(1)	318	2.25	0.28	66	0.036
	(2)	287	1.24	0.19	43	0.028

Within the Poisson model of transmitter release one can obtain an estimate of the mean number of quanta (m) released per presynaptic impulse from the relationship (Martin 1966) $m = \frac{1}{(CV)^2}$ where CV is the coefficient of variation $\left(\frac{SD}{mean}\right)$ of the amplitude distributions of u. EPSPs. A summary of our observations are given in Table 2. The present estimate of m is subject to a number of unproven assumptions and approximations. We shall return to that in the Discussion. Even so it is of interest to mention some of the data of Table II. The first point is the large number of quanta released by each presynaptic impulse. For our best units (15 (1—2)) of which original records have been shown in Fig. 1 m is as high as 100. In three instances we have computed m for different sections of the record from one unit. In two of these the mean amplitude of the unit was rather different (2—2.5 mV and 1.7—0.9 mV) in the two sections. This was undoubtedly due to changes in the recording conditions. The u. EPSPs could be confidently recognized from their threshold of appearance and time course of adaptation. In spite of the changes in mean amplitude of the two u. EPSPs the estimates of m were reasonably consistent (121—110, 79—61).

Within the quantal release theory the average size of each u. EPSP is given by $x = m\,q$ where q is the average change in membrane potential due to the release of a single quantum. The values for q are given in Table II. Corrections for nonlinear summation of quanta has not been applied since even the peak of the unit EPSP

is presumably far from its equilibrium potential The values given for the quantal size (q) deserve some comment Since they are all released by homologous Ia afferent fibres one would expect their size to be approximately constant It is found that they vary with more than a factor of three In the comparison between different cells this may be due largely to differences in membrane potential But it appears that even for different u EPSPs recorded simultaneously in the same cell the quantal size may vary with more than a factor of two (U 15) This can be explained by differences in the location of the synapses with respect to the point of recording presumably within the soma This should be reflected in the time course of the EPSPs As mentioned above there were only minor differences in time courses of the different u EPSPs But as shown theoretically by Rall (1967) even moderate differences in the time course of the EPSP (Fig 3 4) implies appreciable differences in EPSP amplitudes recorded in the soma

Discussion

The large variation in size of u EPSPs is a characteristic feature in DSCT cells The amplitude of the u EPSPs generated by the same group of primary afferent fibres certainly varied over a tenfold range from a fraction of a mV up to about 5 mV By comparison the amplitude of the u EPSPs evoked from the same primary afferents in motoneurones only rarely exceeds 1 mV (Burke 1967 1968) Since these low amplitudes are found also in motoneurones in which the input resistance is higher than in DSCT cells (Burke 1968 Eide *et al* 1969) it is postulated that Ia afferents have a more effective synaptic transmitter action on DSCT cells than on motoneurones The anatomical correlate of the large u EPSPs is presumably the giant synapsis of Szentagothai and Albert (1955) located on the proximal dendrites of the neurone

The demonstration of large u EPSPs in the Clarke s column cells supports the suggestion that the characteristic irregularity of firing of these neurones might be due to such EPSPs produced by impulses in primary afferent fibres converging onto the second order neurone (Jansen *et al* 1966) Recently evidence has been presented that also the much less pronounced irregularity of firing of motoneurones is accounted for by the random occurrence and summation of postsynaptic potentials (Calvin and Stevens 1968)

The estimates of the degree of convergence of primary afferent fibres onto each second order neurone were fairly consistent and suggest that something like one third to one fourth of the primary endings of an ankle muscle projects to each second order neurone The values of Table 1 are based on a simplified model of excitatory synaptic action estimating the number of converging fibres (N) as the ratio of the shunt conductances of the maximum EPSP and the mean u EPSP (Martin 1955) With a slightly more complex model including the membrane capacity in parallel with the membrane resistance N is given by the ratio of the maximum rate of rise of the maximum EPSP and the mean u EPSP (Martin and Pilar 1964) The rate of

change of the membrane potential is less accurately measured than the amplitudes of the corresponding potential deflections For our 'best" cell however the value for the maximum rate of rise of the maximum EPSP was 30 V/sec and the mean value for the u EPSPs was 3 2 V/sec Accordingly the degree of convergence (N) becomes 9 This is considered as reasonably close to the value for this cell (15) in Table I which was 12 It is clear, however, that these methods for the estimation of the degree of convergence will tend to give the minimum number since small EPSPs are more easily overlooked The present numbers can possibly be regarded as estimates of the functional degree of convergence Primary afferent fibres producing even smaller EPSPs than the ones included in the present analysis are presumably equally unimportant for the activation of the cell A recent study suggests convergence to each motoneurone by virtually all Ia afferents from the homonymous muscle (Mendell and Henneman 1968), but the number of functionally significant contacts may be much smaller (Burke 1968)

The tentative explanation of the fluctuations in amplitude of the individual u EPSPs depends entirely on the applicability of the Poisson model of quantal release of transmitter from the presynaptic fibres The usefulness of this model has been convincingly demonstrated at the neuromuscular endplate particularly because that preparation permits a direct measurement of the quantal size (miniature e p p) and the possibility of altering the probability of quantal release by changes in the ionic composition of the bathing solution (del Castillo and Katz 1954, Martin 1966) Evidence for a quantal release of transmitter has been obtained also at a number of neuronal synapses such as the sympathetic ganglia of frog (Blackman Ginsborg and Ray 1963) and chick (Martin and Pilar 1964) the frog spinal cord (Katz and Miledi 1963) the cat motoneurone (Kuno 1964) and the squid stellate ganglion (Miledi 1967) However the question of whether the release of transmitter quanta can be adequately described by the Poisson model also for these synapses has not been finally settled

For the Clarke s column neurones there is at present no possibility of a direct measurement of the quantal size or of experimental alteration of the probability of release of quanta The justification of the present estimates of quantal content is therefore only the possible analogy with these other synapses At synapses of the central nervous system one can think of alternative explanations for the variation in size of the individual unit EPSPs which would invalidate the present estimates of quantal content If for instance each presynaptic fibre supplied many terminal branches to each neurone a variable number of these branches might be invaded by each presynaptic spike Such a system undoubtedly could explain the observed variation of the u EPSP but would require a fairly high degree of presynaptic branching

But also within the Poisson model a number of assumptions which at best are approximations to the physiological situation will influence the estimate of the quantal content The present treatment (Table II) requires that there is a large number of quanta available in the presynaptic terminal and that there is a small probability of release of each of them in response to a presynaptic impulse Further

more, the calculations of Table 2 assume a constant size of the individual q
and they ignore the effect of recording noise on the measured amplitudes
these factors will increase the variability of the u EPSPs amplitudes and therel
crease the CV Accordingly the quantal content has been underestimated Fc
neuromuscular endplate the effect of the variations in quantal size is negligibl
this is not necessarily so in a neurone with the synaptic terminals distributed
a dendrite The importance of these two factors is difficult to assess witho
independent measurement of the quantal size In conclusion the main merit c
present quantal analysis is to suggest the possibility that a large number of q
may be released by each presynaptic impulse This is in contrast to the observ
on the spinal motoneurones at which as little as one quantum may be release
presynaptic impulse in collaterals of the same primary afferent fibres as those
with in the present paper (Kuno 1964) Less commonly larger u EPSPs w
greater quantal content have been observed in motoneurones (Burke and N
1966)

The data of Table II suggest that the average effect of a quantum of rel
transmitter is some 20—40 μV depolarization on the Clarke s column cells
motoneurones the average depolarization of a quantum of transmitter releas
the same Ia afferents has been given as 100 μV (Kuno 1964) but more recer
sults suggest figures as small as 20—30 μV (Jack Miller and Porter 1967 Me
and Henneman 1968) Further experiments are required in order to determin
significance of this apparent similarity in quantal size in motoneurones and D
cells

Compared with motoneurones (Burke 1967) u EPSPs in DSCT cells show 1
less variation in time course It is therefore not likely that the at least tenfold 1
in size of the u EPSPs in individual DSCT cells can be explained exclusive
differential location of Ia synaptic contacts on the somadendritic membrane A
likely explanation is that the difference in size largely depends on the quantal
tent However our results (Table II) can at the most give an indication of su
relationship A much larger material is required to elucidate this question an
this purpose an analysis of small u EPSPs might be particularly valuable

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A Dorso-lateral Spinal Pathway Mediating Information from the Mesencephalon to Dynamic Fusimotor Neurones

By

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Abstract

Appelberg, B and T Jeneskog *A dorso lateral spinal pathway mediating information from the mesencephalon to dynamic fusimotor neurones* Acta physiol scand 1969 77 159—171

In cats anesthetized with halothane, the activity of primary muscle spindle afferents from the long digital flexor muscle of the hind limb was recorded Repetitive electrical stimulation in a restricted part of the mesencephalon in the region of the red nucleus caused a rather selective increase in the sensitivity of the spindles to length changes (dynamic sensitivity) During single shock stimulation in the same region, a response consisting of two positive waves could be recorded in the ventral medulla or in the dorsal part of the lateral funiculus of the spinal cord By making restricted cord lesions it could be shown that the pathway transmitting the mesencephalic influence to dynamic fusimotor neurones was also located in the dorsal part of the

It has been repeatedly demonstrated (Appelberg 1962, 1963, Appelberg and Emonet-Denand 1965, Appelberg and Molander 1967) that the dynamic sensitivity of extensor as well as flexor muscle spindles may be selectively increased by electrical stimulation in the mesencephalon The mesencephalic region effective in this respect comprises the caudal parts of the red nucleus and its immediate surroundings, including the medial longitudinal fasciculus (Appelberg and Molander 1967) A medullary region corresponding well to the inferior olivary nuclear complex was also found to yield spindle effects of a similar nature when electrically stimulated (Appelberg and Molander 1967) This medullary region showed signs of monosynaptic activation when the mesencephalic region was stimulated by single shocks (Appelberg 1967) Such stimuli also evoked a later response in the medulla just ventral to the olive It appeared reasonable to assume that this response represented activity in outgoing fibres from the olivary nuclei It seemed possible that

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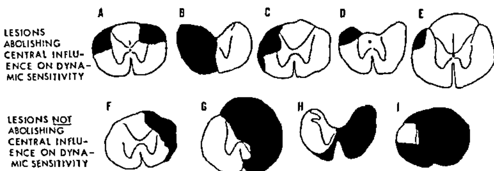


Fig 1 Tracings of series of transverse sections through the spinal cord at C_1—C_2 level Shaded areas indicate lesions made with watchmakers foreceps during the experiments In the experiments represented by tracings B, D and H the dorsal columns were removed during the operations

watchmakers foreceps at the C_1—C_2 level of the spinal cord A number of such lesions are illustrated in Fig 1 In the upper row (A—E) are shown lesions which prevented the MesADC from influencing the dynamic fusimotor neurones In the lower row are lesions, continuously increasing in size from F to I, which did not break this pathway It is evident from the figure that only the dorsal part of the lateral funiculus (DLF) on the left side is essential for the spindle effects to be elicited by right sided mesencephalic stimulation In some experiments, however, it was observed that a dorso-lateral cord lesion on the right side slightly decreased the MesADC influence on the spindle (compare the effect of right sided cord stimulation described in section III a)

II *Response in the dorsal part of the lateral funiculus to contralateral single shock MesADC stimulation*

As described previously (Appelberg 1967) and mentioned in the introduction single shock stimulation within the MesADC caused a long latency response in the medulla just ventrally to the inferior olivary nuclear complex Such a response with a latency of 3 8 msec is shown in the upper record of Fig 2 I To the same stimulus a microelectrode in the DLF of the C_2-segment recorded two positive waves one with a latency of slightly less than one msec (0 8) the other with a latency of 4 3 msec in this case (lower record in I)

Within the whole material considerable variations in late wave latencies were observed The C_2 response was recorded in 21 different cats and its latency varied from 3 2 to 4 5 msec with a mean of 3 9 msec At the medullary level the wave was recorded in 10 cats at latencies from 2 8 to 4 1 msec (mean 3 5 msec) In individual experiments long latencies at the C_2 level generally occurred together with long medullary latencies Occasionally, however the latencies at the two levels could be surprisingly similar and in one case the medullary latency was longer (4 1 msec) than the spinal one (3 9 msec The great variations in response latency in different experiments as well as the last mentioned observation are most likely dependent upon the recording electrodes being placed differently within the fibre bundle sometimes predominantly from coarse fibres, sometimes from thinner ones

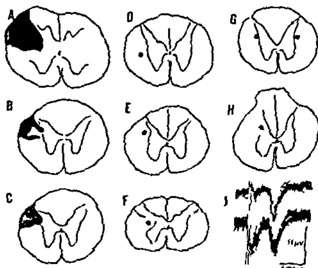


Fig. 2. Tracings of transverse sections through the spinal cord at C_1—C level.
A—C: lesions (shaded areas) abolishing double wave response (see text) at a recording site caudal to the lesion.
D—G: centre of markings (black dots) in electrode sites from which double wave responses were recorded.
In G and in H the black dots also indicate spots from which dynamic sensitivity of spindles could be increased by electrical stimulation. The lesion on the left side in G also diminished the mesencephalic effect on spindles in that experiment. I: 20 superimposed records from ventral medulla (upper) and from DLF of the C_2 segment of the spinal cord (lower) showing responses to single shocks in the MesADC.

That the long latency waves in the medulla and in the cord represented activity in the same pathway was indicated by the following observations.

1. Double shock stimulation in the mesencephalon with varying pulse interval revealed the same characteristic interaction irrespective of whether the medullary or the late spinal wave was studied. Usually the response to the second stimulus was completely inhibited (100 %) during 40—50 msec after the first response but then rapidly grew and became facilitated at around a 70—90 msec interval. A lengthening of the inhibition period during deepened anesthesia was sometimes noticed but this was not further studied.

2. Single shock stimulation in the region of the late medullary response could completely block the appearance of the late spinal wave to mesencephalic stimulation. Such an interaction was observed if the medullary stimulus was introduced 1—4 msec after the mesencephalic one (as will be understood later this was most likely due to collision of antidromic and orthodromic activity in cortico-spinal fibres).

3. A lesion (radiofrequency heating) in the responding medullary region could partially or completely abolish the late spinal wave evoked by MesADC stimulation. Such experiments will be fully described below (sections III b and d, Fig. 4 and 6).

In Fig. 2 spinal lesions abolishing double wave responses (A—C) as well as markings in electrode sites with such responses (D—G) are shown and support the above suggestion that this is a response localized to the DLF.

That the two waves represented activity in spinal tracts rather than synaptic activity in spinal relays was indicated by two facts.

1 A double wave response of a similar nature could be followed through the length of the spinal cord and was thus recorded both in thoracic and lumbar spinal segments In the L_1 region the latencies of the waves were about 2 5 and 8 0 msec (compared with approximate means of 0 8 and 3 9 msec at C level) Conduction distance between C and L_1 may be estimated to be about 200 mm The conduction velocities for the fastest fibres in the two tracts would thus be about 120 m/sec and 50 m/sec respectively Of the two major tracts descending in this part of the cord the fibres of the rubro-spinal tract are known to conduct at velocities of between 41 and 123 m/sec (Tsukahara Toyama and Kosaka 1967) and the fast component of fibres in the cortico spinal tract to have a mean velocity of 50 m/sec (Bishop Jeremy and Lance 1953 Lance 1954)

2 The two waves could be recorded not only by means of electrodes in the cord but also from a dissected and caudally cut dorso lateral fasciculus

III Attempts to prove or disprove the relationship between any of the two waves in the double wave response and the pathway to dynamic fusimotor neurones

a) The spinal cord lesions shown in Fig 2 G do not indicate merely the positions of recording sites of a double wave response in this experiment but also points from which dynamic spindle sensitivity could be increased Such a stimulating effect could also be obtained from the site marked with a black dot in Fig 2 H Furthermore the lesion on the left side in G considerably decreased the stimulatory effect on a muscle spindle from the MesADC on the right side The point on the left side in G indicates the centre of the radiofrequency lesion which was small but the exact size of which was difficult to evaluate

Many experiments were made combining the technique of making cord lesions abolishing the double wave response and simultaneously observing centrally evoked spindle effects Such an experiment is illustrated in Fig 3 Recordings A and B show the spindle s reaction to a linear muscle stretch without (A) and with (B) central stimulation The dynamic sensitivity of the spindle is seen to be strongly increased in B At that stage of the experiment a typical double wave response could be recorded in the dorsal part of the lateral funiculus in the C spinal segment (upper recording to the right) After the C_1 lesion shown in the tracing no change in spindle sensitivity could be elicited (D to be compared with C) and the C_2 double wave response was practically absent (lower record to the right) In no instance was it possible in such experiments to make lesions damaging the pathways conveying the two positive waves without loosing also the MesADC influence upon spindle dynamic sensitivity

In many of these experiments the effect of different depths of anesthesia on cord responses and on central spindle influence was also studied In such studies the short latency spinal wave was shown to be of no importance for the MesADC spindle effects This wave revealed itself to be very resistant to anesthesia while the central effects on spindles were always abolished even by moderate deepening of the anesthetic level Such a sensitivity to anesthesia was however also noted for the long latency spinal wave and in fact it may be said that anesthesia induced disappearance

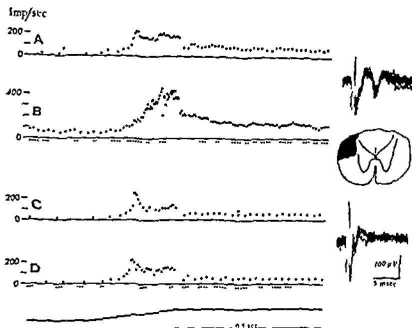


Fig 3 A—D, Instantaneous frequency recordings from primary muscle spindle afferent from the long digital flexor muscle before during and after 4 mm extension of muscle at about 8 mm/sec A and C are controls while B and D were obtained during repetitive stimulation in the MesADC In A and B the spinal cord was intact, C and D were recorded after the DLF lesion shown in the tracing to the right The lesion also abolished (lower superimposed record to the right) the double wave response recorded caudally to the lesion (upper record to the right) The period of extension is indicated by the oblique line below recording D

of the late spinal wave constantly occurred simultaneously to a disappearance of MesADC influence on spindles

b) At this stage of the investigation it seemed reasonable to assume that the short latency spinal wave was due to activation of the rubro-spinal tract It also appeared likely that the long latency spinal wave recorded during single shock MesADC stimulation represented activity in the pathway from the mesencephalon to dynamic fusimotor neurones Attempts were made, therefore, to localize the extent of the area showing a late wave response in the medulla Possibly, by such a procedure, the course of the tract at this level would be revealed and perhaps the expected additional relay in the pathway found The outcome of one such experiment is demonstrated in Fig 4 On the dorsal view of the brain stem in B is shown the position of tracks with (filled circles) and without (open circles) a late wave response It can be seen that the response could be recorded throughout the entire length of the brain stem from a rostral pontine level to the beginning of the spinal cord Characteristically, the response crossed the midline at the transition between medulla and cord Histological controls showed the crossing to occur exactly at the level of the pyramidal tract crossing In A which is a tracing of a histological section through the rostral medulla at the level indicated by the thin horizontal line in B, the course of one of

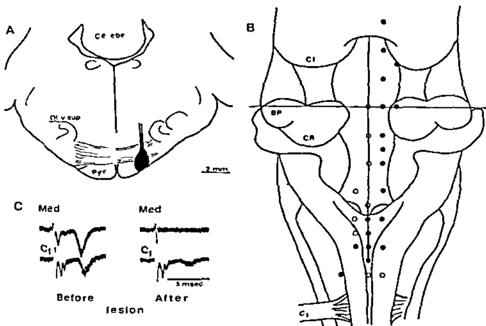


Fig 4 B is a dorsal view of the cat's brain stem (cerebellum removed) Filled circles indicate tracks from which a late wave response of the type shown in C_{II+I} could be recorded Open circles indicate tracks without such response

A is a tracing of a transverse section at the level indicated by the thin horizontal line in B Part of the most lateral track at this level is seen with a lesion made in the centre of the responding region.

C shows superimposed records obtained in medulla and in the first cervical segment before and after the lesion in A

Abbreviations Cerebell Cerebellum Ol v sup Oliva superior Pyr pyramid Med Medulla C_1 first cervical segment CI colliculus inferior BP brachium pontis CR corpus restiforme

the tracks is shown together with the position as well as the extent of a thermo lesion made in a point with a typical late wave medullary response (recording Med in C) The centre of the lesion is in the pyramid and the lateral part of this structure is destroyed by the lesion This led to a strong diminution of the late wave as recorded at a C_1 level (compare lower recordings marked C_1 before and after lesion in C)

c) It was thereby clear that the late wave in the medulla and in the cord represented activity not in olivary efferents but rather in pyramidal tract fibres It seemed unlikely though that the mesencephalic stimulus could spread as far as to the cerebral peduncle The long latency of the response already at pontine and medullary levels also seemed to furnish further evidence against such a direct activation of pyramidal tract fibres In a few experiments therefore responses to MesADC stimulation were looked for in the cerebral cortex and the effect of cortical cooling or ablation on the late spinal wave was tested Such an experiment is illustrated in Fig 5 The mesencephalic stimulus caused a typical double wave response in the C_1 segment (A_1) The latency to the second wave was in this case 3 8 msec The same

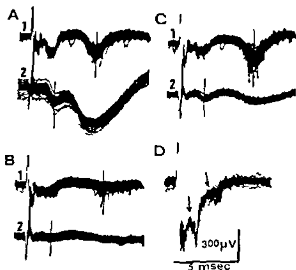


Fig 5 A_1 20 superimposed records from the DLF of the C segment
A same from the sensori motor cortex close to the lateral end of the cruciate sulcus
B_1 and B similar recordings obtained during cooling of the cortex with icecold Ringer s solution
C_1 and C similar recordings obtained during rewarming of cortex.
D superimposed records from the DLF of the C segment during single shock stimulation of the cortical area recorded from in A—C

stimulus also caused an evoked potential in the cerebral cortex in the region of the cruciate sulcus (A_2) This response consisted of two positive negative waves the first of which was succeeded by the second one so quickly that its negative phase was usually concealed The latencies measured to the start of each wave were 1 5 and 3 0 msec It is noteworthy also that the cortical and spinal responses were elicited from strictly the same depths in the mesencephalic stimulating electrode track The stimulating threshold for a detectable cortical response was only slightly lower than that for a detectable late C wave During application of ice cold Ringer s solution to the cortex the cortical response was considerably diminished the second wave being relatively more influenced than the small first one (Fig 5 B and C_2 Note that the decreased response size is partially due to short circuiting by the applied Ringer) The cortical cooling also caused a diminution or disappearance of the second spinal wave (Fig 5 B_1)

During recovery from cortical cooling the distance between the two cortical waves was seen to be increased the latency of the second one amounting to about 3 8 msec while that of the first one was unchanged A comparable lengthening of the latency of the late spinal wave to 4 5 msec was simultaneously recorded In Fig 5 D a double wave response (arrows) recorded in the DLF of the C segment to stimulation of the cerebral cortex is shown The waves appear with latencies of 1 and 2 5 msec

d) From the functional point of view i e with regard to mesencephalic influence on muscle spindle behaviour the experiments employing cortical cooling were of considerable interest It was constantly observed that *the cooling could completely block the transmission of the late spinal wave without influencing the effect of repetitive mesencephalic stimulation on spindles* This was the first experimental observation indicating that the late spinal wave caused by single shock MesADC stimulation

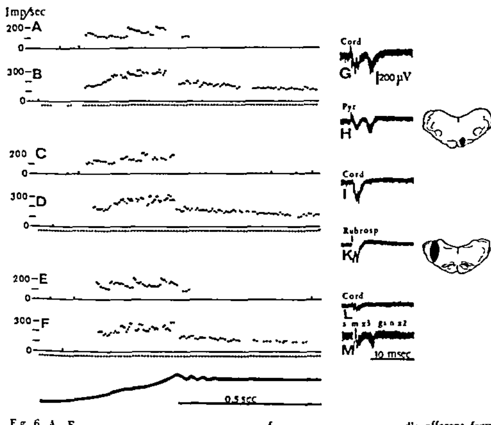


Fig 6 A ... dle afferent from the MesADC

G double wave response in C_4 to single shock MesADC stimulation
H late medullary wave recorded in centre of lesion shown in upper tracing
I C_4 response after lesion Late wave is absent
K, recording from the rubro-spinal tract in medulla in centre of lesion shown in lower tracing)
L, C_4 recording after this second lesion Only traces of rubro-spinal tract wave remains
M same as L but stimulus strength and gain of amplifier increased

might after all not be due to synchronous activation of the tract directed to dynamic fusimotor neurones

To add further proof to the view that neither the short latency (insensitive to anesthesia) nor the long latency (sensitive to cortical cooling or ablation) waves in the spinal response to MesADC stimulation actually had anything to do with the pathway under investigation experiments of the type illustrated in Fig 6 were performed This experiment started by demonstrating a typical spindle influence from the mesencephalon (B to be compared with the control A) Single shocks applied

to the mesencephalic stimulating spot caused a double wave response *in the DLF in C_2 (G)* An electrode was then placed in the *medulla in a position* where the medullary late wave was recorded (H) *A lesion* was made around the tip of that electrode This resulted in the disappearance of *the late wave in C_2 (I)* while the mesencephalic influence on the spindle was *unchanged (C, D) In the* upper tracing of a section through the medulla *it is seen that* the lesion partially destroyed the pyramid on the *right side The* medullary electrode was then moved to a lateral *position in the medulla* on the left side, where the rubro spinal tract is known to *pass The* recording of a rubro-spinal tract response is seen *in K and the extent of* the lesion made in *this region* is *demonstrated* in the lower tracing to the right After the lesion *the early wave in C* was practically absent (L) When the strength *of stimulation* was increased three times and the gain of the amplifier doubled only *traces of the* two waves could be seen (M) Following the rubro-spinal lesion the spindle was influenced to the same extent as previously (E F)

IV *Spontaneous variations in dynamic sensitivity*

As previously reported (Appelberg and Molander 1967) *the level of control dynamic sensitivity* of the spindles may differ considerably between different experiments and also change during an experiment It was recently shown that such spontaneous variations in the activity of dynamic fusimotor neurones may be induced from the brain and are thus independent of segmental reflex activity (Appelberg and Jeneskog 1968) It was repeatedly observed in the present experiments that the descending pathway responsible for this variable influence upon the dynamic fusimotor system does not descend in the dorsal *part of the lateral funiculus. Spontaneous* variations in dynamic sensitivity were thus often observed following *cord lesions* bilaterally destroying the dorsal parts of the lateral funiculi (cf Appelberg and Jeneskog 1968 Fig 1 C)

Discussion

The experiments described succeeded in localizing a tract directed to dynamic fusimotor neurones to the dorsal part of the lateral *funiculus On the other hand the experiments failed* to give any information concerning the course of the tract at a medullary level or to establish a connection between the tract and an anatomically defined descending system Two major systems descending in this part of the cord i e the rubro-spinal and cortico-spinal tracts could be eliminated as being of importance for the contact between the mesencephalon and the dynamic fusimotor system Vedel (1966) found that lesions in the medullary pyramid abolished cortical influence upon dynamic fusimotor neurones Appelberg and Molander (1967) considered it possible that such pyramidal lesions might have influenced also the inferior olive which was shown by them to similarly activate the dynamic fusimotor system It can still not be excluded that the two systems are in fact identical but studied at different levels by the different authors This would mean that the

stimulation of the sensory motor cortex might act via the red nucleus and the inferior olive The other possibility namely that the mesencephalic stimulus should influence the spindles via the sensory motor cortex and the cortico-spinal tract seems to be excluded by the present experiments

Only two other descending tracts are known to pass in the dorso-lateral part of the cord namely the so called dorsal reticulo-spinal pathway demonstrated by physiological methods (Engberg Lundberg and Ryall 1965 1968 a) and a monoaminergic system emanating from the raphe nuclei (Brodal Taber and Walberg 1960 Dahlstrom and Fuxe 1965 Engberg Lundberg and Ryall 1968 b) It seems reasonable therefore to pursue the present work by paying special attention to these two known systems It cannot be excluded however that an additional hither to unknown dorso-lateral system exists Stimulation seemingly localized to the inferior olive also caused an increased dynamic sensitivity of spindles (Appelberg and Molander 1967) It was observed then and has been repeatedly noticed that even quite small lesions in the olivary region seriously reduced the mesencephalic influence on spindles

Appelberg and Emonet Denand (1965) found that mesencephalic stimulation also ipsilaterally to the spindles used for recording was effective in influencing the dynamic behaviour of the spindles In the present experiments only contralateral stimulation was employed but on the other hand it was observed that stimulation in the dorso lateral parts of the cord (*cf* Fig 2 G) could evoke identical spindle effects from either side As mentioned in section I in the results it was also occasionally observed that dorso-lateral cord lesions on the right side slightly diminished mesencephalic effects on the spindles The double wave cord response to mesencephalic stimulation was sometimes seen on the right side also (Fig 2 G) However this was probably due to current spread to the other side of the mesencephalon since it disappeared when the stimulating electrode was moved slightly more laterally From this it seems reasonable to assume that the connection between the mesencephalon and the dynamic fusimotor neurones is a crossed one Probably however the influence of each tract at the segmental level is bilateral When due to current spread the mesencephalon was bilaterally stimulated right sided cord lesions therefore slightly reduced the effects on the spindles

The long latency positive wave that could be recorded in the medulla or in the dorsal part of the lateral funiculus of the spinal cord was proved to be transmitted via the cerebral cortex It is difficult to interprete the disappearance of the wave during cortical cooling or ablation in any other way The exact nature of this transmission cannot be revealed however without further experiments It is to be expected though that either of two principally different mechanisms is responsible

1 Axons of small pyramidal tract (PT cells are known to send collaterals to the cells of the red nucleus (Tsukahara Fuller and Brooks 1967 1968 Excitatory collaterals from small PT cells are also given to large PT cells Phillips 1959 Armstrong 1965 Brooks and Asanuma 1965 Takahashi Kubota and Uno 1967) A stimulus applied in the red nucleus might therefore antidromically invade the

axons of the small PT cells This would lead to an excitation of the large PT cells also, the axons of which would then transmit the volley to medullary and spinal levels The distance from the red nucleus to the cortex is about 20 mm and the mean conduction velocity of small PT cell axons has been estimated to be 14 m/sec (Bishop, Jeremy and Lance 1953, Lance 1954) One synapse on the large PT cells *may be expected to require a maximum of 0 7 msec* The mean conduction velocity in large PT cell axons is about 50 m/sec (same authors as above) and the distance from cortex to medulla is about 40 mm This would mean an ascending conduction time of 1 4 msec, a synaptic delay of 0 7 msec and a descending conduction time of 0 8 msec (compare with the observed latency of 1 msec from cortex to C_4 in Fig 5 D), *i e* a sum latency of 2 9 msec which is considerably less than the measured mean of 3 5 msec

2 *At least one major ascending system* passes in the immediate vicinity *of the red* nucleus, or rather through the nucleus, namely fibres from the cerebellum destined for the red nucleus itself and for the ventro lateral nucleus of the thalamus (VL) (Jansen and Jansen 1955) These fibres have been shown to conduct at a velocity of 10—20 m/sec (Uno, Yoshida and Hirota 1967 as cited in Eccles Ito and Szentagothai 1967) They are also known to make monosynaptic contact with the VL cells If the axons of such cells have similar conduction velocities and monosynaptically contact *large PT cells* a total loop time" from the red nucleus to the medullary pyramid of about 3 6 msec may be estimated This is very close to the observed mean

It was somewhat surprising to find that when rubro spinal and cortico spinal waves were abolished by suitable lesions, no response to MesADC stimulation remained in the DLF This may be explained in different ways One possibility is of course that the recording electrode being placed in a position for maximal recording of rubro-spinal and cortico spinal waves, did not record activity in a tract with a somewhat different *location Provided that the tract to dynamic fusimotor neurones* is polysynaptic this would also reduce the possibilities of activating it by single shocks Another possible explanation remains namely that the MesADC stimulus acts not through a tract acting by itself on the dynamic fusimotor system but *instead* perhaps by releasing activity in another descending tract

It is of considerable interest also to once more stress the fact that spontaneous centrally induced variations in dynamic sensitivity of spindles may still occur after *bilateral DLF lesions This indicates that there is at least one more system regulating* the activity of dynamic fusimotor neurones a system descending more ventrally in the cord In fact Bergmans and Grillner (1967 1968) have demonstrated the existence of a monoaminergic inhibitory control of dynamic fusimotor neurones Spontaneous variations in such a system may perhaps occur under the conditions of the present experiments

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Elicitation of Reflex Vagal Relaxation of the Stomach from Pharynx and Esophagus in the Cat

By

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Abstract

Abrahamsson, H and G Jansson *Elicitation of reflex vagal relaxation of the stomach from pharynx and esophagus in the cat* Acta physiol scand 1969 77 172—178

Distension of different parts of the esophagus in chloralosed cats caused prompt and pronounced relaxation of the stomach Even slight distension was sufficient to elicit gastric relaxation, and the degree of relaxation varied with that of the esophageal distension The gastric relaxatory response was shown to be due to elicitation of a vago-vagal reflex to the stomach The response could be e
vagotomy It could *not*
relaxation obtained by
stomach It is conclude

recorded in connection with pharyngeally
xation pattern described plays an essential
deglutition

A previous study of the reflex control of gastric motility revealed the existence of a vago-vagal relaxatory reflex (Jansson 1969) Electric stimulation of afferent vagal fibres at near supradiaphragmatic or cervical levels produced pronounced reflex relaxation of the stomach The efferent pathway of this reflex was the vagal high-threshold "relaxatory" fibres first described by Martinson (1965 b) These efferent fibres whether activated reflexly or direct, elicit gastric relaxation even after administration of atropine or guanethidine It was thought that the relaxatory fibres might be involved in "receptive relaxation" of the stomach, *e g* in connection with swallowing

The present study was undertaken to explore whether receptor mechanisms in pharyngeal and esophageal parts of the gastro-intestinal tract are capable of producing reflex gastric relaxation via the vagal high-threshold relaxatory fibres

Material and methods

28 cats weighing 2 5—4 9 kg were deprived of food for 24—36 hrs before the expts After induction with ether they were anesthetized with chloralose 40—60 mg/kg b w

The method used for recording gastric motility has been described previously (Jansson 1969) Stomach volume was recorded at constant low intragastric pressure (4 to 8 cm H_2O) with a large rubber balloon *via* an esophageal catheter and a volume reservoir connected to a float recorder The volume of the emptied balloon was about 15 ml which is included in the figures below

For distension of different parts of the esophagus another rubber balloon 2 5—7 cm long (in some expts adjustable in position) was placed round the polyvinyl catheter fitted to the stomach rubber balloon By means of a reservoir the esophageal balloon could be distended to a variable extent with simultaneous recording of its volume changes *via* a connection between the reservoir and a float recorder The lowest intra esophageal pressure recorded in anesthetized cats was —4 to —5 cm H_2O during quiet breathing (Botha 1959 and personal observations) The intra-esophageal pressure was measured from a zero reference level placed about 5 cm below the level of the esophagus of the supine cat The esophagus was usually kept distended 1—2 min (range 4 sec to 20 min)

The pharynx was mechanically stimulated by touching the mucosa with a forceps or a glass rod Deglutition was repeatedly induced by such a tactile stimulation or by applying 0 4—0 6 [illegible]

electrodes were connected to a Grass Stimulator S4 Stimulations of 0 1—20 imp/sec at 5—12 V and a pulse duration of 0 1—4 msec were used

To eliminate the reflex gastric responses to esophageal and pharyngeal stimulation either both vagal nerves were cut or temporarily blocked by cooling to 0° C This was performed by placing about 1 cm of the cervical segment of the nerve in a groove of a metal tube which was perfused with ice water

In four experiments the spinal cord was transected between C6 and C7 and in two the spinal medulla was anesthetized by subdural administration of 1 5—2 ml of 2 % lidocaine (Xylocain® Astra) through a fine polyethylene catheter

The blood pressure was recorded by a mercury manometer connected to a catheter placed in a femoral artery Atropin [illegible]
0 2—1 mg/kg b w in a to[illegible]
CIBA) was given in a dose [illegible]
(Flaxedil® May & Baker) [illegible]
movements Artificial respiration was then maintained by a respiration pump

Results

Esophageal distension Inflation of the esophageal balloon with 2—20 ml of air or water (Fig 1) produced a pronounced relaxatory response of the stomach within 5—7 sec. It could not be blocked by atropine but was eliminated by vagotomy

Fairly moderate distension of the esophagus was usually sufficient to elicit these reflex gastric relaxations *e g* an increase of the pressure in the esophageal balloon to 2—5 cm H_2O above the reference level (see Methods) Stepped increases of the esophageal distension correspondingly increased the gastric relaxation (Fig 2) Even quite local esophageal distension with a small balloon in the cervical upper or lower thoracic segment was efficient. Furthermore even a brief distension lasting only a few seconds produced a clear gastric relaxation Upon continuous esophageal distension for 10—20 min the stomach remained relaxed until the balloon was deflated (Fig 2)

The reflex gastric relaxations were mostly considerable in magnitude (see Fig 2)

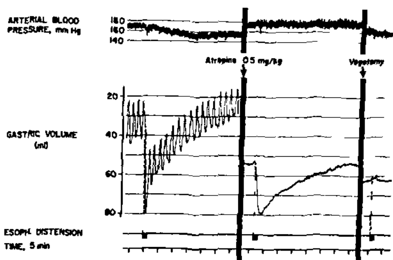


Fig 1 Cat 3 0 kg Gastric volume recorded with a balloon method at constant pressure of 8 cm H_2O Esophageal distension 35 cm H_2O by inflation of 18 ml into the esophageal balloon Reflex gastric relaxation on esophageal distension before (left panel) and after (middle panel) blocking of cholinergic excitatory nervous activity with atropine (0 5 mg/kg b w) Reflex relaxation persisted after atropine but was no longer obtainable after acute bilateral vagotomy at cervical level

Thus a 10—15 cm H O increase in esophageal pressure for 1—2 min regularly produced a 30—60 ml increase of gastric volume *i e* the gastric volume could be more than doubled (Fig 2) The reflex relaxations persisted after administration of atropine (≤5 mg/kg b w) as well as after guanethidine in doses known to block adrenergic reflex effects on gastric motility (Jansson and Martinson 1966) They also persisted after spinal transection or spinal anesthesia The same was true when Flaxedil® was given to eliminate reflex movements of skeletal muscles

In all experiments however the reflex gastric relaxations disappeared after cervical or supranodal transection of both vagal nerves (Fig 1 3) or by cooling of the vagal nerves to 0° C In the latter case they could be reproduced after rewarming the nerves

After complete vagotomy gastric relaxations which exhibited the characteristics of the reflex responses could be induced by electric stimulation of the *distal* ends of the divided vagal nerves with impulses strong enough to activate the efferent relaxatory fibres to the stomach (Fig 2—4)

Pharyngeal stimulation Mere touching of the mucous membrane in the pharynx with a forceps or a glass rod elicited prompt gastric relaxation in lightly anesthetized cats (Fig 3) The mechanical stimulation usually induced single or repeated deglutitions thereby induced gastric relaxations were superimposed upon each other until the last swallow (Fig 4) *In some animals spontaneous swallowing movements* were observed to be accompanied by gastric relaxation Furthermore repetitive

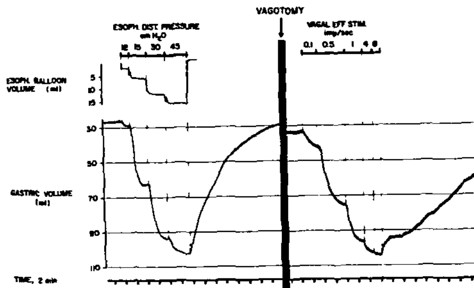


Fig 2 Cat 4 1 kg Atropine 0 5 mg/kg
Left panel Gradually increasing stomach relaxation reflexly induced by graded esophageal distension
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swallowing movements followed by superimposed gastric relaxatory responses could be elicited by applying 20 % ethanol to the pharynx All these relaxations persisted after atropine but not after vagotomy though deglutition could then still be observed (Fig 4)

Discussion

The present study demonstrated that mechanical stimulation of the pharynx and esophagus induced reflex gastric relaxations These relaxations resembled those produced by afferent vagal stimulation (Jansson 1969) That the reflex elicited by esophageal distension was of vago-vagal nature was evident from the fact that the response could still be produced after exclusion of all spinal connections but was abolished by vagotomy

It is further clear that the vagal relaxatory nerve fibres Jansson and Martinson 1965) constitute the efferent pathway for the reflex studied These fibres elicit a gastric relaxation which persists after cholinergic blocking agents and sympathicolytic

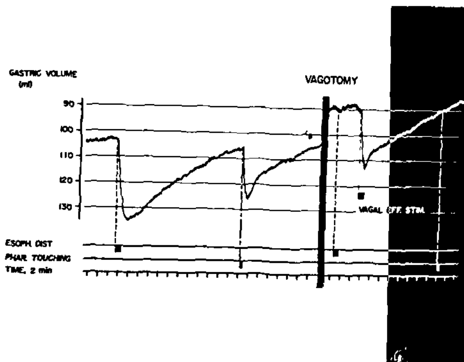


Fig 3 Cat 2 5 kg Atropine 0 5 mg/kg

Left panel Reflex stomach relaxation induced by touching the mucous membrane of aboral parts of the pharynx with a forceps and by esophageal distension (15 cm H_2O) Swallowing movements were observed on mechanical pharyngeal stimulation

Right panel These responses were no longer demonstrable after vagotomy immediately cranial to nodose ganglion Stomach relaxation induced by stimulation of the distal ends of the divided vagal nerves (8 imp/sec 2 msec, 8 V)

drugs (Martinson 1965 a, Campbell 1966) which also characterized the reflex gastric relaxations presented in this study Moreover this reflex response closely mimicked that obtained by direct stimulation of the vagal relaxatory fibres in all other respects (Fig 2, *cf* Martinson 1965 a) It is therefore concluded that these reflex gastric relaxations—elicited by mechanical stimulation of the pharynx and the esophagus and observed in connection with swallowing movements—are in the efferent direction mediated by the high-threshold vagal relaxatory fibres

The vagal nerves are known to convey *afferent* nerve fibres from *all parts* of the esophagus (Kuntz 1953 p 207) and such fibres probably constitute the afferent link for the reflex gastric relaxations induced from the esophagus Swallowing can however also be elicited by *pharyngeal stimulation of regions with afferent nerves* in the glossopharyngeal and trigeminal nerves (for ref see Doty 1968, p 1874) It is therefore possible that at least part of the afferent fibres activated during *pharyngeal stimulation and swallowing* belonged to these nerves instead of to the vagal ones

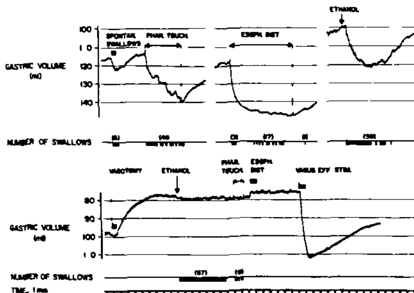


Fig 4 Cat 3 0 kg Atropine 0 5 mg/kg

Upper panel [illegible]

Lower panel After "low cervical vagotomy similar stimuli elicited repetitive swallows but without gastric relaxation Electric efferent stimulation of the divided vagal nerves (4 imp/sec 2 msec 8 V) produced relaxatory gastric response Esophageal distension 20 cm H O 14 ml

Activation of afferent vagal fibres by esophageal distension presupposes mechanoreceptors and in the rat esophagus Andrew (1956 1957) demonstrated sensory endings that caused afferent discharge on esophageal distension and during propulsive esophageal contractions It appears likely that such receptors are involved in the esophageo-gastric vago vagal relaxatory reflex

The mode by which the reflex gastric relaxations were elicited in the present study suggests that they constitute an integral part of the complex autonomic co-ordination of deglutition. Thus it was shown that swallowing movements were accompanied by reflex stomach relaxation The phenomenon of gastric receptive relaxation is old knowledge but little or nothing has so far been known about the nervous mechanisms involved Cannon and Lieb (1911—12) observed gastric relaxation during swallowing in the unanesthetized cat already when the bolus passed cervical esophageal parts and Veach (1926) observed gastric relaxation upon esophageal distension. Lind *et al* (1961) reported fundus pouch relaxation in the dog during the act of swallowing The present findings strongly suggest that gastric receptive relaxation during normal swallowing is predominantly or only a consequence of an activation of the high threshold vagal relaxatory fibres to the stomach

Another important aspect of gastric "receptive relaxation" is the ability of the stomach to adapt its volume to increasing amounts of food (*cf* Grey 1917—18) In earlier experiments (Jansson 1969) a vago-vagal reflex gastric relaxation was induced by stimulating afferent vagal fibres, at least some of which probably originated in the stomach wall However, adequate activations of possible gastric receptors involved in such 'gastro-gastric" reflexes cannot be demonstrated with the technique used in the present study, but such investigations are in progress

Th [illegible] (Grant B68- [illegible] 1 (052) 7[illegible] [illegible] Force from [illegible] ulty of Medicine University of Goteborg

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The Effect of Marked Hyperventilation upon Tissue Levels of NADH, Lactate, Pyruvate, Phosphocreatine, and Adenosine Phosphates of Rat Brain

By

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Abstract

GRANHOLM, L., L. LUKJANOVA and B. K. SIESJÖ. *The effect of marked hyperventilation upon tissue levels of NADH, lactate, pyruvate, phosphocreatine, and adenosine phosphates of rat brain.* Acta physiol. scand. 1969. 77. 179—190.

In order to study the effect of pronounced hyperventilation upon the redox state of brain tissue rats were hyperventilated with air and measurements were made of the cortical level of NADH using the microfluorometric method of Chance. Control measurements were made of the tissue concentrations of lactate, pyruvate, ATP, ADP, AMP, and phosphocreatine during and after the hyperventilation. It was found that when the rats were hyperventilated to arterial pCO_2 levels below about 20 mm Hg, the NADH fluorescence invariably increased. Although part of this increase may represent a hemodynamic artefact due to a decrease in the tissue blood volume, a large part of it should represent a true reduction in the NADH/NAD system. With hyperventilations of this degree there was a proportionally larger increase in lactate than in pyruvate and thus an increased lactate/pyruvate ratio, but there were no changes in phosphocreatine, or in adenosine phosphates, except for a small but significant increase in the AMP concentration. The NADH increase during hyperventilation was slightly decreased when pure oxygen was administered and completely normalized when 3—5 % CO_2 was given. The experiments thus strongly suggest an increased reduction in the NADH/NAD system of the brain during pronounced hyperventilation.

We have recently reported that hyperventilation to arterial pCO_2 values below 25—20 mm Hg leads to an increased lactate/pyruvate ratio in the cerebral cortex, and in cisternal cerebrospinal fluid (CSF) of cats, as well as to an increased NADH level in cortical tissue of rats (Granholm and Siesjö 1968, 1969, Granholm, Lukjanova and Siesjö 1968). These results strongly suggest that pronounced hyperventilation leads to tissue hypoxia provided hypoxia is defined as an increased reduction in one of the main oxidoreduction systems of the electron transport chain. However, such an interpretation of the results was complicated by the fact that the ATP/ADP and

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the phosphocreatine/creatine ratios of the cat cerebral cortex were normal or near normal (Granholm and Siesjö 1969 see also Hohorst Betz and Weidner 1968)

The present paper gives a full account of the measurements of cortical NADH levels in the rat, but in order to elucidate the matter further tissue concentrations of lactate pyruvate phosphocreatine and adenosine phosphates were measured in the same species under identical experimental conditions The results strongly indicate that *pronounced hyperventilation leads to an increased reduction in the* NADH/NAD system and it will be discussed if this reduction can be interpreted as a hypoxic equivalent in spite of the fact that the concentrations of the energy rich phosphates were upheld

Methods

Male rats of the Wistar strain *weighing 300—400 g were anesthetized with i p phenobarbital* (150 mg/kg) and tracheotomized Six animals were anesthetized with 70 % N_2O and 30 % O_2 after induction with divinylether Ventilation was maintained with a Palmer miniature respirator and the animals were immobilized with tubocurarine chloride (Tubocurarin Vitrum) i p The O_2 and CO_2 concentrations of the inspired gas were altered by delivering gas mixtures of known composition to the respirator One femoral artery was cannulated for blood sampling and for blood pressure recording with an electro manometer (Elema Stockholm) *Blood was anaerobically drawn from this cannula during the experiments and analysed* for pH pCO_2 and pO_2 at 37° C with the help of microelectrodes (Radiometer Copenhagen and Eschweiler and Co Kiel) A 20—30 mm² area of the parietal cortex was exposed with a dentist s burr through a longitudinal skin incision The dura was left intact and it was carefully controlled that a blood free field was obtained

For the recording of the kinetics of oxidation reduction changes of intracellular NADH the microfluorometric method described by Chance *et al* (1962) was used The signal from *the photomultiplier was amplified and registered by a chart recorder The excitation energy* from a 1kW mercury arc was used filtered through a 366 mμ primary filter Two secondary filters (Wratten types 2 C and 47 A) gave a broad pass band (400—500 mμ) peaking at 450 mμ The microscope was focused on an area containing a minimum of visible blood vessels Optical fields of 300 μ were used A long focus lens (working distance about 3 cm) was used so that small changes in the working distance due to the motion of the brain with changes in blood volume did not seriously affect the intensity In order to further minimize *the effect of tissue movement the focus was adjusted so that it fell slightly below the cortical* surface The fluorescence changes were calibrated by comparing the deviations observed in the actual experimental situation with the total deflection obtained during the administration of nitrogen gas Thus the increase in fluorescence from the baseline to the point of cessation of cardiac activity was taken as the standard reference and all other fluorescence changes were calculated as a percentage of this standard reference However the fluorescence increased further after the moment of cardiac arrest in an approximately exponential fashion For those *experiments in which this anoxic plateau was recorded* the fluorescence changes could be calculated as a percentage of the maximum increase in anoxia (see Results and Discussion

Since the fluorescence recorded is sensitive to changes in the blood content of the tissue 5 control experiments were made in order to test the effect of gross changes in the blood content of the tissue In these experiments one carotid artery was ligated and cannulated for subsequent injections of a Krebs Henseleit solution in order to flush the blood away from the ipsilateral hemisphere Before this was done the NADH level was recorded during the *administration of nitrogen gas When the anoxic plateau value for* NADH was approached the tissue was flushed with the haemoglobin free solution the new NADH value was recorded and the tissue was then illuminated and inspected ensuring that the pial vessels were visually emptied from blood

4 groups of rats were used to study the lactate pyruvate ATP ADP AMP and phosphocreatine concentrations in the supratentorial parts of the brain at specific times during or after pronounced hyperventilation In all groups the tissue was frozen by pouring liquid *nitrogen into a plastic funnel* fitted into the longitudinal skin incision (Pontén 1966) One group in which no craniotomy was made served as a control series In another group the brain was frozen after 10 min of pronounced hyperventilation with air and in the remaining

Results

The main object of the present experiments was to study the oxidoreduction state of the NADH/NAD system of the rat brain in pronounced hyperventilation by means of direct NADH recordings (I), and by means of lactate/pyruvate measurements (II) In order to allow an interpretation of the fluorescence signals control measurements were made by administering pure oxygen or CO-containing gas mixtures, or by studying the effect of flushing the tissue with a haemoglobinfree solution In order to facilitate an interpretation of the tissue lactate and pyruvate concentration changes the tissue concentrations of phosphocreatine and of adenine nucleotides were measured

I Fluorometric in vivo measurements of the tissue NADH levels

The tissue NADH levels were recorded in 50 rat experiments after preliminary methodological trials in about 25 animals In the following the results obtained in the nitrous oxide and in the phenobarbital series will be described together since there were no significant differences between the groups In each animal, one to three periods of hyperventilation were carried out In two of the animals pure oxygen was administered during the hyperventilation and in 10 expts, 3—5 % CO_2 was given during continuous mechanical overventilation

As mentioned in the method section all fluorescence changes were compared with the increase in fluorescence at the moment of cardiac arrest during nitrogen breathing In 15 expts this increase in the NADH level was compared to the total increase in NADH during nitrogen administration In these experiments the increase in fluorescence at the moment of cardiac arrest amounted to 27—91 per cent of the total increase in fluorescence during anoxia with a mean of 60 per cent A typical example of this is shown in Fig 1 which shows that the NADH level increased abruptly 10—15 sec after the nitrogen breathing was started In all these recordings there was a temporary break in the NADH curve which coincided with a fall in blood pressure, and a secondary rapid rise in NADH when the blood pressure had declined to very low levels, approaching a plateau value approximately 10 min after the onset of the administration of the nitrogen gas

A The effect of hyperventilation Fig 2 shows a typical series of events during repeated hyperventilations to an arterial pCO below 15 mm Hg During the first hyperventilation the NADH increased by 34 per cent of the NADH increase obtained at the moment of cardiac arrest during nitrogen breathing (arrow) while the second and third hyperventilations led to NADH increases of 33 and 27 per cent of the reference deflection After the first hyperventilation there was a pronounced overshoot of the base line lasting almost 15 min while the second hyperventilation

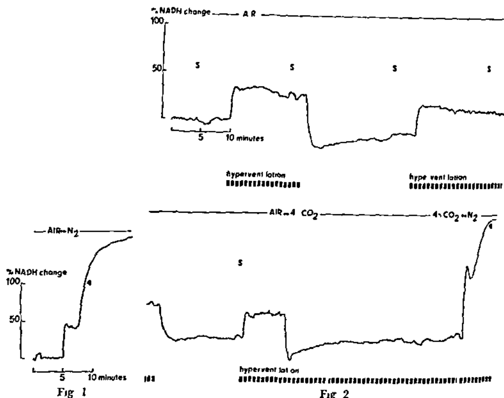


Fig 1

Fig 2

Fig 1 NADH fluorescence recorded through the intact dura in a phenobarbital anesthetized and immobilized rat The figure shows the increase in fluorescence obtained when the inspired gas was changed from air to N_2 Arrow indicates cardiac arrest In this and in most of the following figures the NADH increase from the baseline in air breathing to that obtained at the moment of cardiac arrest was taken as the standard reference deflection (about 60 % of the total NADH increase in anoxia)

Fig 2 Increase in brain surface NADH fluorescence in three consecutive periods of hyperventilation (denoted by interrupted course lines) During the first two hyperventilations the animal was breathing air During the third hyperventilation, 4—5 % CO_2 was administered during continuous mechanical overventilation, causing a return of the fluorescence to the baseline Note overshoot of fluorescence after the first hyperventilation and after the start of the CO_2 administration The sampling of arterial blood gave irregular increases in the fluorescence (S, see text)

was followed by a much less pronounced overshoot During the third run mechanical hyperventilation was maintained when 4 % CO_2 was administered This led to a complete normalization of the NADH level after a transient overshoot of the base line The end of the experiment shows the increase in fluorescence obtained during administration of nitrogen gas with the point of cardiac arrest indicated (arrow)

The effects of hyperventilation on the NADH levels have been summarized in Table I, which lists the blood parameters and the NADH changes calculated as a percentage of the standard reference deflections It can be seen from the table that the NADH increase during the hyperventilation was around 25 per cent of the

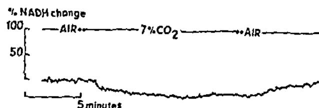
TABLE I The changes in NADH concentration of rat cerebral cortex as studied by spectrofluorometry during repeated hyperventilations and intermediate periods of normal ventilation. The periods of hyperventilation lasted on the average 14 min and the intermediate periods on the average 16 min. Means ± SEM are given. The arterial pCO_2 and pO_2 are expressed in mm Hg, the actual bicarbonate concentration in mEq/l plasma and the NADH changes in per cent of the change from the baseline to the value obtained at the cessation of cardiac activity during nitrogen administration

	n	pH	pCO_2	Act HCO_3^-	pO_2	NADH incr %	NADH restitution	Maximal "over shoot" %
Control values	14	7.402 ±0.01	33.5 ±1.2	20.0 ±0.7	85.0 ±3.6	—	—	—
First hyperventilation	14	7.707 ±0.02	12.6 ±0.5	15.8 ±0.5	96.9 ±5.0	24 ±3	—	—
First intermediate period	11	7.392 ±0.02	27.6 ±1.1	17.4 ±0.7	90.5 ±5.5	—	Complete in 10 of 11	18±8 in 10 of 11
Second hyperventilation	10	7.682 ±0.02	12.4 ±0.2	14.4 ±0.8	96.8 ±6.5	29 ±4	—	—
Second intermediate period	6	7.409 ±0.02	32.7 ±5.1	20.7 ±2.4	83.7 ±6.9	—	Complete in 5 of 6	14±5 in 3 of 6
Third hyperventilation	5	7.642 ±0.01	11.4 ±0.1	12.0 ±0.4	85.9 ±2.9	28 ±8	—	—

standard reference level irrespective of if the period of hyperventilation was the first, second or third in the same experiment. Since the NADH increased to a fairly stable plateau value during the hyperventilation the calculated values approximate steady state values. The listed overshoots of the base line after the hyperventilations represent the maximum values obtained soon after the resumption of normal ventilation, since most of the overshoots normalized within 10—15 min.

B The effect of CO_2 administration. In 10 expts 3—5 % CO_2 was administered during the hyperventilations, examples of which are shown in Fig. 2 and 3. In 4 expts 7—8 % CO_2 was given in normocapnic conditions. Fig. 3 illustrates that these concentrations led to clearcut decreases in the NADH concentrations recorded. Table II summarizes these experiments showing that 3—5 % CO_2 during hyperventilation normalized the NADH recorded while 7—8 % CO_2 in normocapnia led to a mean decrease in the NADH concentration recorded of about 40 %.

C The effect of O_2 administration. The effect of administration of pure oxygen is shown in Table II and illustrated in Fig. 4. When oxygen was given in normocapnia (mean arterial pO_2 about 85 mm Hg) there was a small but consistent decrease in the NADH concentration recorded, which on the average amounted to



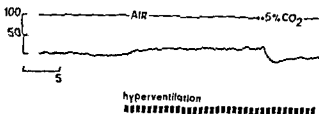


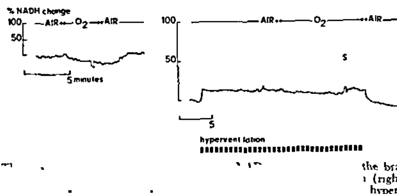
Fig. 3. Effect of CO_2 administration (5—8 %) on the NADH fluorescence of brain cortex in a rat during normal ventilation (upper tracing) and during continuous hyperventilation (lower tracing).

16 per cent of the standard reference deflection. This effect of oxygen was confirmed under hyperventilation in two experiments.

D. The effect of gross changes in the blood volume. As described in Methods the effect of changes in the tissue blood volume was studied by flushing the tissue with a haemoglobin free solution when the tissue NADH concentration was approaching the anoxic plateau value. In two of the original five experiments the flushing seemed to remove all the blood from the field of inspection and the effect

TABLE II. The changes in the NADH concentration of rat cerebral cortex as studied by spectrofluorometry during administration of oxygen or carbon dioxide. The induced changes were studied both in normal ventilation and in hyperventilation. The NADH changes are expressed in per cent of total change from baseline to the increase at the cessation of cardiac activity during administration of nitrogen gas. Arterial pCO_2 and pO_2 are expressed in mm Hg.

	n	Gas concentration per cent	Mean pCO_2	Mean pO_2	NADH decrease per cent
Effect of O_2 during normal ventilation	7	100	36.4	323.6	16
Effect of O_2 during hyperventilation	2	100	8.8	373.7	7
Effect of CO_2 during normal ventilation	4	7—8	70.7	97.0	38
Effect of CO_2 during hyperventilation	10	3—5	31.5	130.0	Return to baseline in 9/10. Overshoot max 18 in 7/10



... the brain surface
... (right tracing)
... hyperventilation

of flushing could be compared to both the effect of pronounced hyperventilation and to the NADH deflection in anoxia. In these experiments the NADH concentration recorded increased by 21 and 29 %, respectively, of the *total* increase in the NADH during anoxia (see Discussion) while the effects of pronounced hyperventilation in the same experiments were 32 and 20 %, respectively. These results are illustrated by Fig. 5.

E. The effect of blood sampling. Fig. 2 and 4 show that irregular increases in the tissue NADH concentrations were obtained each time an arterial sample (S) was withdrawn (see also the previous figure in Granholm, Lukjanova and Siesjö 1968). Since the sampling of arterial blood was found to be associated with a small decrease in blood pressure, two control experiments were made with measurement of the arterial blood pressure during the sampling of the usual 3—4 glass capillaries. It was then found that this sampling procedure resulted in a fall of blood pressure of 15—35 mm Hg to a minimum level of 60 mm Hg. The pressure was then normalized 3—5 min after the sampling.

II. Enzymatic measurements of tissue metabolites

Table III shows the results of the measurements of the concentrations of lactate, pyruvate, phosphocreatine and adenosine phosphates during and after a period of

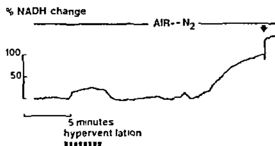


Fig. 5. The effect of flushing the brain with a haemoglobinfree solution on the NADH fluorescence recorded from the brain surface of a rat. The animal was first hyperventilated and then subsequently made anoxic by means of administration of nitrogen gas. When the fluorescence was approaching a plateau value, the tissue was flushed and it was verified by inspection that the tissue was free of blood.

TABLE III Concentrations and ratios of lactate, pyruvate, ATP, ADP, AMP and phosphocreat ne study The metabolite concentrations are given in mmoles/ kg of wet tissue Per cent significant ($p<0.001$) increase of La Py and La/Py, and an increase of AMP, statisti changes gradually diminished during the subsequent normal ventilation Hyperventila 0 01)

	n	pCO_2	Hb	% H_2O
Controls	6	38 6 ±1.2	16 0 ±0 3	78 5 ±0 3
10 min after onset of hyperventilation	6	14 7 ±0 9	17 6 ±0 6	77 0 ±0 4
3 min after termination of hyperventilation	6	29 7 ±0 7	17 4 ±0 3	78 8 ±0 3
15 min after termination of hyperventilation	6	29 7 ±0 7	17 1 ±0 9	78 6 ±0 3

10 min of hyperventilation as compared to a control group in which no hyper ventilation and no craniotomy had been performed No control group with craniotomies was made since other studies from the laboratory (Kjallquist Nardini and Siesjo 1969) have confirmed the increased lactate/pyruvate ratio and the increased AMP concentration in the rat brain during marked hyperventilation The table shows that although the lactate and pyruvate concentrations and the lactate/pyruvate ratios increased appreciably during the hyperventilation there were no significant changes in the other metabolites except for a slight increase in the tissue AMP concentration It was particularly noticeable that the tissue phosphocreatine and ADP concentrations were unchanged in spite of a reduction of the arterial pCO to below 15 mm Hg It should also be noted that there were no significant changes in the tissue ADP concentration 3 min after the termination of the hyperventilation i e at the point where the NADH measurements revealed an overshoot of the baseline (see Discussion)

Discussion

There are a number of experiments which indicate that hyperventilation leads to tissue hypoxia in the brain Thus it is well known that hyperventilation reduces the cerebral blood flow amounting to a decrease of about 50 per cent with extreme degrees of pCO reduction (Kety and Schmidt 1946 Alexander et al 1965) Concomitant to this decrease in blood flow there is a decrease in the relative oxygen tension measured in cortical tissue (Sugioka and Davis 1960 Meyer and Gotoh 1960) It has been repeatedly discussed if this decrease in blood flow, and the decrease in oxygen tension are associated with a true hypoxic state in the tissue There is some indication that the function of the tissue is changed since hyperventilation is

in the rat brain during variations in ventilation comparable to those in the spectrofluorometric water refers to wet brain tissue Means ± SEM are given — Hyperventilation caused a highly cally significant at the 5 per cent level (0 05 > p > 0 02), as examined with Student's t test These tion also seemed to cause a decrease of tissue water, significant at the 2 per cent level (0 02 > p >

La	Py	ATP	ADP	AMP	PCr	La/Py	ATP/ADP
0 748 ±0 027	0 071 ±0 005	2 77 ±0 03	0.28 ±0 01	0 10 ±0 01	5 40 ±0 14	10 8 ±0 7	9 8 ±0 4
3 340 ±0.356	0 198 ±0 013	2 75 ±0 04	0.28 ±0 02	0 15 ±0 02	5 28 ±0 09	16 8 ±1 0	10 0 ±0 6
1 714 ±0.256	0 112 ±0 011	2 85 ±0 03	0 32 ±0 01	0 12 ±0 01	5 47 ±0 12	15 2 ±1 8	8 9 ±0 4
1 189 ±0 058	0 080 ±0 004	2 74 ±0 06	0,28 ±0 01	0 12 ±0 01	5 39 ±0 09	14 9 ±0 6	10 0 ±0.5

accompanied by a slowing of the EEG (Davis and Wallace 1942) However, none of the studies quoted has given unequivocal proof that hyperventilation leads to a lowering of the oxygen supply of the tissue of such an order that tissue hypoxia is present. Recently, Cohen Alexander and Wollman (1968) have shown that pronounced hyperventilation in man leads to an increased glucose uptake and to an increased lactate production of the brain These results, as well as those presented by Plum and Posner (1967) by Leusen and Demeester (1966) and by us (Granholm and Siesjo 1968 1969) show that hyperventilation is associated with an increased rate of glycolysis in the tissue but this is no conclusive proof of the presence of tissue hypoxia since alkalosis has been found to increase the rate of glycolysis without apparent signs of an interference with the oxygen consumption in brain and muscle tissue (Domonkos and Huszak 1959 Scheuer and Berry 1967) However, the fact that the augmented glycolysis of the tissue in hyperventilation decreases under hyperbaric conditions (Reivich *et al* 1968) more strongly suggests that the lactate production is partly of an anaerobic nature

The present experiments have confirmed our preliminary reports (Granholm Lukjanova and Siesjo 1968), in showing that pronounced hyperventilation leads to an increased tissue lactate/pyruvate ratio and to an increased NADH fluorescence from the rat cerebral cortex. These results thus seem to provide conclusive evidence of an increased reduction in the NADH/NAD system and thus possibly of tissue hypoxia (see below) However before we can accept these findings to prove the existence of hyperventilation hypoxia thus defined we must discuss if the methods used involve artifacts of such a nature that they complicate the interpretation.

Measurements of the tissue NADH concentration with the microflu tric method of Chance *et al* (1962) involve the possible source of error

changes in the amount of haemoglobin in the field of measurement. Thus it is well known that haemoglobin absorbes a substantial part of the excitation energy and of the fluorescence emission (Schnitger *et al.* 1965, Chance and Schoener 1965). This factor has been investigated by Chance *et al.* (1962) who computed that about 23 per cent of the fluorescence is absorbed by blood in the brain, a figure which fits well with those measured in our control experiments (see Results and Fig. 5). This means that part of the fluorescence changes recorded in hyper- and hypocapnia, represent hemodynamic artefacts due to an increased hemoglobin concentration in hypercapnia, and a decreased hemoglobin concentration in hypocapnia. It is also possible that the fluorescence increases recorded during the sampling of arterial blood (see Results) is at least partly due to such hemodynamic factors but the lactate/pyruvate changes measured by Kjällquist, Siesjö and Zwetnow (1968) indicate that a transient hypoxia may be involved in even very moderate decreases of the arterial blood pressure.

There are strong reasons to believe that the fluorescence changes recorded in hyper- and hypocapnia at least in part represent true changes in the redox state of the NADH/NAD system. Thus, although a part of the fluorescence decrease during *hypercapnia* may be due to an increased blood volume, concomitant to the increased cerebral blood flow, the results obtained with administration of pure oxygen in normocapnia indicate that the increased oxygen supply during hypercapnia involves a real oxidation of NADH. In *hypocapnia* the fluorescence increase recorded amounted to 15—20 % of the maximal deflection in anoxia (see Results), while a complete outflushing of the blood from the tissue may increase the fluorescence by 20—25 % (Chance *et al.* 1962 and present results). *It* is not known if the reduced blood flow during pronounced hyperventilation will give any significant reduction of the *volume* of blood in the field under inspection, but it is improbable that the blood volume will be reduced by more than 50 %. We thus have to conclude that part of the fluorescence increase in pronounced hyperventilation involves a true increase in the NADH concentration.

The conclusion that pronounced hyperventilation leads to an increased reduction in the NADH/NAD system is fully confirmed by the measured increase in the tissue lactate/pyruvate ratio. Thus, it has been repeatedly pointed out that the lactate/pyruvate and the NADH/NAD systems are coupled in a steady state *in vivo* (*Bücher and Klingenberg 1958, Huckabee 1958, Hohorst 1960, Williamson, Lund and Krebs 1967*). However, since the steady state reaction between the two redox systems involves hydrogen ions the true cytoplasmatic NADH/NAD ratio can only be calculated if the hydrogen ion concentration is known (*cf.* Tobin 1964). In a previous paper we have shown that the lactate/pyruvate ratio changes in accordance with the changes in the tissue hydrogen ion concentration in hypercapnia, but that the increased lactate/pyruvate ratio in pronounced hyperventilation is in discordance with the hydrogen ion dependent equilibrium between the redox systems, *i.e.* that it indicates an increased NAD reduction (Granholm and Siesjö 1969). This conclusion is supported by the findings of Leusen, Weyne and Demeester

(1968, and Leusen personal communication) who showed that *in vitro*, when the circulation factor is absent the increased glycolytic rate at low CO tensions (and thus at alkalotic pH values) was accompanied by a decreased lactate/pyruvate ratio

It thus appears proven beyond doubt that pronounced hyperventilation is accompanied by an increased tissue NADH/NAD ratio However, such an increase cannot immediately be equated with tissue hypoxia If it is agreed that tissue hypoxia is best defined as an increased reduction in the important redox systems of the tissue, and if the NADH/NAD system is considered to be a good index of the redox situation in the electron transport chain the present results obviously indicate that pronounced hyperventilation is accompanied by tissue hypoxia However if it is agreed that tissue hypoxia is not manifest until the tissue stores of energy rich phosphates are being depleted (see Hohorst Betz and Weidner 1968) the present results show an increased NAD reduction but not tissue hypoxia The small increase found in the present experiments in the tissue AMP concentration during hyperventilation is indicative (*cf* Chance *et al* 1965, Minard and Davis 1962) but cannot give full proof of hypoxia Until further experiments have been carried out this important question must evidently be left open Probably such experiments should involve a study of other cytoplasmatic and mitochondrial redox systems and should also be directed towards possible regional changes in metabolism

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Changes in the Activity of Isolated Vascular Smooth Muscle in Response to Reduced Osmolarity

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Abstract

JONSSON, O *Changes in the activity of isolated vascular smooth muscle in response to reduced osmolarity* Acta physiol scand 1969 77 191—200

The effects of hypo-osmolarity on the spontaneous activity of the smooth muscle in the isolated portal vein of the rat have been studied in solutions with varied ionic composition A comparison between the stimulatory effects of lowered osmolarity and increased [K]o indicated

fact that the membrane potential of smooth muscle is more dependent on the transmembrane concentration gradient for potassium at higher levels of external potassium concentrations

The stimulatory effects of hypo-osmolarity are markedly reduced at a low [Na]o but are not considerably affected by comparable reductions in [K]o or [Cl]o This indicates that the transmembrane sodium ion gradient is involved in the mechanisms by which hypo-osmolarity excites the spontaneous activity of vascular smooth muscle One possible interpretation is that the swelling of the muscle cells in hypotonic solutions causes an increase in the permeability of the cell membrane to the relatively larger sodium ion

In an earlier study concerning the effects of extracellular osmolarity on the activity of vascular smooth muscle it was reported that procedures which could be expected to make the muscle cells swell increase their spontaneous activity and *vice versa* (Johansson and Jonsson 1968) It was proposed that these effects were not a consequence of changes in the ionic concentration gradients across the cell membranes but also due to variations in the membrane permeabilities to the different ions The present investigation describes in more detail the effects of reduced osmolarity on the spontaneous activity of the rat portal vein The ionic mechanisms behind these effects were studied by varying simultaneously the extracellular osmolarity and the concentrations of sodium potassium and chloride ions

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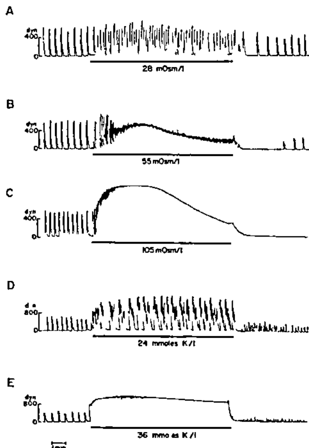


Fig 1 Isometric recordings of mechanical activity in isolated rat portal vein Responses to lowered osmolarity (A—C) and to increases in the potassium ion concentration (D—E)

described in Fig 1 2 and 3 below were performed in order to elucidate the role of the $[K]_i/[K]_o$ ratio in the responses of the portal vein to lowered osmolarity

Mechanical responses of the portal vein to different degrees of hypo osmolarity and to increases in $[K]_o$ respectively are shown in Fig 1

A decrease in osmolarity by 28 mOsm/l (Fig 1A) resulted in an increased frequency of the contractions but their amplitude remained almost unchanged The higher degree of hypo-osmolarity —55 mOsm/l applied in Fig 1B resulted in a sustained incomplete tetanus the peak tension of which hardly exceeded that in the control period These responses may be compared with that produced by the four fold increase in $[K]_o$ shown in Fig 1D where an increase in the amplitude and duration as well as in the frequency of the contractions were seen The positive chronotropic effect is more pronounced in B than in D indicating that a reduction in osmolarity by 55 mOsm/l exerts a stronger membrane excitation than the four fold increase in $[K]_o$

A contracture (see further below) developed when the osmolarity was reduced by

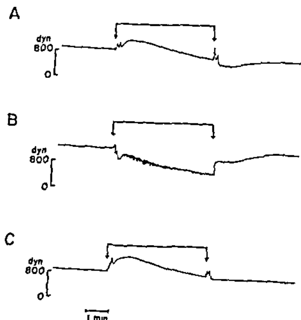


Fig 2 Effects of hypo osmolarity of variations in $[K^+]_i$ on a contrac caused by a 6 fold increase in potassium ion concentration (36 K^+/l)

A Reduction in the osmola corresponding to minus 30 mm NaCl/l at a $[K^+]_i$ of 36 meq/l

B Reduction in the osmolarity responding to minus 30 mm NaCl/l at a $[K^+]_i$ of 18 meq/l

C Increase in $[K^+]_o$ from 36 me to 54 meq/l

105 mOsm/l (Fig 1C) It should be noticed, however, that within some minutes t contracture began to return towards the level of passive tension The sluggish ons of the excitations seen in Fig 1A—C depends on the fact that the muscles had be exposed to an NaCl low solution containing sucrose before the periods of hyp osmolarity The sucrose thus had to diffuse out from the extracellular space befo the hypo-osmotic solution could influence the smooth muscle cells It appears fro the uptake curve of sucrose-^{14}C in rat portal vein (Arvill Johansson and Jonsso 1969) that this elimination of sucrose from the extracellular space should be practica ly complete within two min This agrees well with the fact that the maximum ex citation was developed within a couple of minutes after the change from the sucrose substituted to the hypo-osmotic, NaCl-low solution

Fig 1E shows that a 6-fold increase in $[K]_o$ also gave a contracture tension o about the same magnitude as that initially obtained in Fig 1C (note the difference in calibration), but the potassium contracture was well sustained in contrast to that produced by hypo-osmolarity If the cells behaved like perfect osmometers in the ex periment of Fig 1 there would be a maximal decrease in $[K]_i$ by about 37 per cent in response to the largest of the reductions in osmolarity (C) Thus it is evident that the decreases in $[K]_i/[K]_o$ produced by the reduced tonicity in Fig 1A–C are small when compared to those resulting from increases in $[K]_o$ (D—F) The excitatory responses to hypo-osmolarity cannot therefore be simply attributed to the induced changes in the potassium gradient

The experiments presented in Fig 2 and 3 were performed in order to study the effects of hypo-osmolarity at a high level of $[K]_o$ At the beginning of the recording

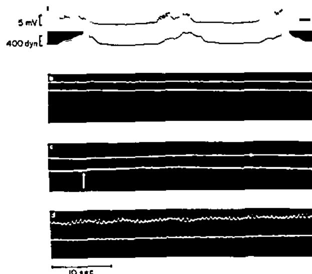


Fig 3 Simultaneous recording of electrical and mechanical activity in rat portal vein
a Spontaneous activity in normal Krebs solution
b Tenth min in an isotonic Krebs solution with 36 meq K⁺/l 62 mmoles NaCl/l and 60 mmoles sucrose/l
c Change (arrow) from the solution in 3b to a medium of the same ionic composition but from which the sucrose was removed
d Seventh min in a hypo-osmotic Krebs solution (minus 30 mmoles NaCl/l) with 18 meq K/l

in Fig 2A the muscle had been exposed for 10 min to an iso-osmotic solution containing 36 meq K/l i e about 6 times normal and it was therefore in a state of sustained contracture During the period indicated the preparation was exposed to a solution of the same ionic composition but with osmolarity reduced by 55 mOsm/l (see Methods) It can be seen that the response to this reduction in osmolarity was a transient increase in the contracture tension

In Fig 2B the same degree of hypo-osmolarity as in A was matched against a simultaneous decrease in [K] from 6 times to 3 times the normal Before and after the period indicated the muscle was exposed to the same isotonic solution as during the control period in Fig 2A When the osmolarity and the potassium ion concentration were simultaneously reduced in Fig 2B the inhibitory effect of the decrease in $[K]_o$ seemed to overshadow the stimulatory effect of reduced osmolarity as judged by the fact that the contracture was replaced by an incomplete tetanus

The effect on the contracture tension of an increase in $[K]_o$ from 36 meq/l to 54 meq/l is shown in Fig 2C It should be noticed that the augmentation of the contracture produced by this 50 per cent increase in $[K]_o$ is quite comparable to that produced by the hypo osmolarity in Fig 2A

Fig 3 which is taken from a sucrose gap experiment illustrates the electrical and mechanical responses to reductions in osmolarity at high potassium concentrations similar to those of the experiment in Fig 2 The spontaneous activity in normal Krebs solution is shown in Fig 3a The phasic contractions were well correlated with spike potentials occurring in bursts which lasted for 5 to 10 sec In b the muscle had been exposed for 10 min to a solution with the same composition as that used in the control periods of Fig 2 *i e* an isotonic solution with 6 times the normal potassium ion concentration The cell membranes were depolarized no action potentials appeared and a contracture had developed When as in Fig 2A the osmolarity of the

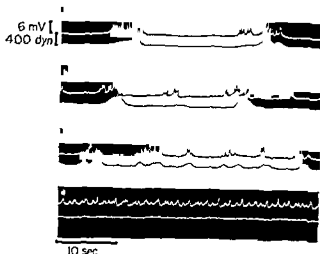


Fig 4 Effects of hypo osmolarity at a low (c) and at a more normal NaCl concentration (d)
a Normal Krebs solution
b Tenth min in an approximately isotonic Krebs solution with 32 mmoles NaCl/l and 180 mmoles sucrose/l
c Fifth min in a hypo-osmotic Krebs solution (242 mOsm/l) with 32 mmoles NaCl/l and 120 mmoles sucrose/l
d Fifth min in a hypo-osmotic Krebs solution (229 mOsm/l) with 92 mmoles NaCl/l

solution was reduced by approximately 60 mOsm/l (arrow in Fig 3c), the membrane potential was somewhat further depolarized and the contracture tension augmented However, when the same degree of hypo-osmolarity was applied together with a reduction in $[K^+]_0$ from 6 to 3 times the normal (Fig 3d), there was a repolarization, spikes reappeared and phasic contractions replaced the contracture (compare Fig 2B)

The results reported in Fig 2 and 3 indicate that the effects on muscle activity of hypo-osmolarity and of increased $[K^+]_0$, respectively are quantitatively more comparable when these variations in the environment are superimposed on an initially high level of $[K^+]_0$ than when such variations are induced at normal $[K^+]$, (Fig 1)

It was suggested in an earlier study (Johansson and Jonsson 1968) that the inhibitory effects on the portal vein of increased osmolarity was not simply mediated via changes in the concentrations of intracellular ions but also by variations in the membrane permeabilities to the ions A relatively greater decrease in the permeability to sodium than to potassium would help to explain the marked inhibition of activity in hyperosmotic solutions The experiments presented below were designed to determine whether the sodium ion was of special importance for the effects of reduced osmolarity observed in the present study If a change in the membrane permeability to Na^+ were an essential factor in the response to hypo-osmolarity one would expect that such responses would be relatively weaker in Na^+-deficient solutions (see further below) Fig 4 shows a sucrose gap recording from an experiment performed on the basis of this argument The spontaneous activity of the preparation in normal Krebs solution is illustrated in Fig 4a In b the muscle had been exposed for 10 min to a solution which was approximately isotonic but contained only 32 mmoles NaCl/l The pattern of activity after 10 min in this solution was quite comparable to that in the control period When the osmolarity of this medium was reduced by 60 mOsm/l the frequency of spike potentials and contractions increased to a very small extent

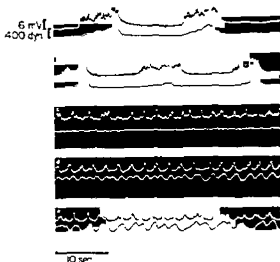


Fig 5 Effects of hypo-osmolarity at a low Cl concentration (c) and effects of increases in [K]o at a low and normal NaCl concentration (d e)
a Normal Krebs solution
b Tenth min in an isotonic Krebs solution with 32 mmoles NaCl/l and 90 mmoles sodium ethanesulphonate/l
c Fifth min in a hypotonic Krebs solution with 32 mmoles NaCl/l and 60 mmoles sodium ethanesulphonate/l
d Fifth min in an isotonic Krebs solution with 32 mmoles NaCl/l 162 mmoles sucrose/l and 24 mmoles KCl/l
e Fifth min in an isotonic Krebs solution with 104 mmoles NaCl/l and 24 mmoles KCl/l

(Fig 4c) This moderate excitation caused by hypo-osmolarity at the low extracellular NaCl concentration should be compared with the much more conspicuous stimulation of the smooth muscle seen in Fig 4d where a comparable reduction in osmolarity was induced at a NaCl concentration of 92 mmoles/l In the latter case the muscle exhibited continuous spike firing and an incomplete tetanus The results shown in Fig 4 thus lead to the conclusion that hypo-osmolarity excites the smooth muscle to a degree which is greatly dependent on the prevailing level of Na and/or Cl in the medium

The experiment illustrated in Fig 5 a—c was performed in order to determine wether the inability of the hypo-osmolarity to stimulate muscle activity in Fig 4c was due to the low sodium or to the low chloride ion concentration Record a shows the activity in normal Krebs solution In b the muscle had been exposed for 10 min to a Krebs solution in which 90 mmoles NaCl/l were replaced by equimolar amounts of sodium ethanesulphonate *i e* this solution had a normal [Na] but a [Cl] which was reduced to the same level as in Fig 4c The muscle activity in Fig 5b was quite comparable to the control state (a In c the sodium ethanesulphonate concentration was reduced by 30 mmoles/l which should give a similar reduction in osmolarity as the solutions applied in Fig 4c and d The excitation produced by hypo-osmolarity in this Cl low solution resembled that obtained in Fig 4d where [Cl] was closer to normal It does not seem therefore as if the Cl concentration is of critical importance for the effects of lowered osmolarity

To exclude the possibility that the portal vein is generally inexcitable in Na deficient solutions the stimulatory effect of a four fold increase in [K]o was tested in an isotonic medium containing only 32 mmoles NaCl l Fig 5d The response obtained under these conditions is quite similar to that seen in 5e where the potassium concentration was increased four times at a more normal concentration of NaCl

It thus seems as if the smooth muscle can be depolarized and mechanically activated by potassium in a normal manner at low $[Na^+]_o$ whereas hypo osmolarity turned out to be rather ineffective under such circumstances

The possible influence of a low $[K^+]_o$ on the stimulatory effects of reduced osmolarity were examined in some experiments It was then found that the excitation produced by a decrease in osmolarity by 55 mOsm/l at a $[K^+]_o$ of 1 meq/l was of about the same intensity as that caused by this degree of hypotonicity at a normal $[K^+]_o$ Thus it seems as if the potassium ion concentration is less important than the sodium ion concentration for the effects of hypo osmolarity on the spontaneous activity

Discussion

The response of vascular smooth muscle activity associated with increased cell volume may be of pathophysiological interest as it has been shown that, in hypertensive disease, the vessel walls contain more water than normal Such water-logging has been found in the renal arteries of patients with essential hypertension and in arterioles of rats with experimental hypertension (Tobian and Binion 1952, Tobian 1961, Tobian *et al* 1961) Tobian and Binion discuss the possible influence of this passive swelling of the vascular wall on the blood flow resistance The demonstration of a stimulatory effect of swelling of the muscle cells on the spontaneous activity in isolated vascular smooth muscle suggests that the observed water logging in the arteries of hypertensives may lead to an increase in vascular tone and perhaps in vascular reactivity

The mechanical responses to lowered osmolarity illustrated in Fig 1 show that hypo-osmolarity primarily enhances the frequency of the contractions while an increase in [K] increases both the amplitude and the frequency of the contractions It should be noticed that the duration of each compound contraction was longer when the muscle was exposed to increased $[K]_o$ than to hypo-osmolarity At more drastic reductions in osmolarity tension development is impaired as shown in Fig 1C where the contracture tension declined towards the passive tension level after only a few minutes In experiments where electrical and mechanical activity were recorded simultaneously depolarization of the cell membranes and cessation of spike activity were seen during the development of the contracture in such hypo-osmotic media but repolarization could not be demonstrated at the decline of the contracture These observations are in agreement with those of Blinks (1965) concerning the contractile responses of frog skeletal muscle in different aniso-osmotic solutions He found that the tetanus tension was considerably reduced in solutions with markedly lowered osmolarity

The excitatory responses obtained in the present study at more moderate reductions in osmolarity (for instance -55 mOsm/l) were however well sustained even for prolonged periods of exposures

A comparison between the excitation caused by increased [K] and by hypo-osmolarity reveals that a reduction in osmolarity by about 20 per cent (Fig 1B) has

a more marked stimulatory effect on the spontaneous activity than a four fold increase in $[K^+]_o$ (1D), and that a reduction in osmolarity by 37 per cent (1C) has about the same effect as a six fold increase in $[K^+]_o$ (1E) Provided the smooth muscle cells swelled like perfect osmometers in the hypotonic solutions $[K^+]_i$ would decrease in proportion to the reduction in extracellular osmolarity It is evident from the comparison in Fig 1 that the effects of lowered osmolarity on muscle activity at 'normal' levels of $[K^+]_o$ are not entirely mediated *via* changes in the $[K^+]_i/[K^+]_o$ ratio

On the other hand when similar experiments were performed at an initially high level of $[K^+]_o$ a 20 per cent reduction in osmolarity caused a weaker excitation than a 50 per cent increase in $[K^+]_o$ (Fig 2A and C) and the depolarizing effect of this degree of hypo-osmolarity was outbalanced by a simultaneous lowering of $[K^+]_o$ by 50 per cent (Fig 3) This change in the relation between the effects of increased $[K^+]_o$ and of hypo-osmolarity when the potassium ion concentration in the control situation is increased from the "normal" 6 meq/l to 36 meq/l may be attributed to the fact that the membrane potential of smooth muscle is more intimately related to $[K^+]_i/[K^+]_o$ at the high levels of $[K^+]_o$ (Holman 1959, Marshall 1962, Axelsson *et al* 1967) A similar increase in the importance of the potassium concentration gradient on the responses to increased osmolarity at higher potassium ion concentrations was found in an earlier study (Johansson and Jonsson 1968)

An additional factor might be that the sodium chloride concentration was somewhat lower in the experiments where the effects of reduced osmolarity were tested at a higher $[K^+]_o$ than at the normal $[K^+]_o$ The experiments illustrated in Fig 4 and 5 showed that the excitation caused by a reduction in osmolarity was strongly dependent on the actual sodium ion concentration in the solution The excitatory response to hypo osmolarity was not significantly impaired in Cl^- deficient (Fig 5c) or K^+-deficient solutions but it seemed to be critically dependent on $[Na^+]_o$ The stimulatory response to increased potassium ion concentration in an isotonic NaCl low medium (Fig 5e) shows that the muscles are not generally inexcitable at low $[Na^+]_o$

The membrane potential of smooth muscle is considered to be influenced not only by the equilibrium potential for the potassium ion but also by the equilibrium potentials for sodium and chloride ions The net effects of these 3 ions on the membrane potential (E_m) are expressed in the "constant field equation"

$$E_m = \frac{RT}{F} \log \frac{[K^+]_i + \alpha [Na^+]_i + \beta [Cl^-]_o}{[K^+]_o + \alpha [Na^+]_o + \beta [Cl^-]_i}$$

where α and β represent the membrane permeabilities for sodium and chloride ions respectively relative to that for potassium ions (Goldman 1943) An increase in the cell water caused by reduced extracellular osmolarity would lower the intracellular ion concentrations and this in turn would depolarize the cell membrane provided that β is not too large

However, as discussed above the dramatic effects on the spontaneous smooth

muscle activity of even small reductions in osmolarity makes it doubtful that these responses could be mediated entirely *via* changes in the intracellular ion concentrations. An additional factor might be changes in the properties of the cell membranes in hypotonic media. It is conceivable that an enlarged cell volume might alter the structure of the cell membrane by stretching in such a way that the permeabilities to the different ions would increase particularly if the passage is thought to be through a system of pores. The ion that would be most influenced by such a change is the largest of the permeable ions, i.e. the hydrated sodium ion. An increase in α on exposure of the smooth muscle to hypo-osmotic solutions, would according to the '*constant field equation*', depolarize the membrane and thus contribute to the stimulatory effect of these solutions.

The fact that $[Na^{+}]_{o}$ seems to be of special importance for the stimulatory effects of reduced osmolarity fits well into the hypothesis that one of the mechanisms by which hypo-osmolarity excites the vascular smooth muscle is by increasing the membrane permeability to the sodium ion.

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Changes in the Cell Volume of Isolated Vascular Smooth Muscle in Response to Reduced Osmolarity

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Abstract

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The intracellular water of smooth muscle from the rat portal vein decreases on exposure to hyperosmotic solutions to an extent expected for perfect osmometers (Arvill, Johansson and Jonsson 1969). The purpose of the present study has been to investigate the variations in cell water produced by reductions in extracellular osmolarity.

After 15 min of incubation at 37° C in a solution which was 105 mOsm/l hypo-osmotic (minus 60 mmoles NaCl/l) compared to normal Krebs solution, the intracellular water increased by 14±7 per cent in muscles stretched to their *in situ* length and by 35±9 per cent in unstretched muscles. When the same procedures were performed at 1° C, a temperature that considerably reduced the mechanical response to hypo-osmolarity, the volume of the cell water in the stretched and unstretched muscles increased to approximately the same extent (40 and 35 per cent respectively). These results suggest that the active tension development associated with excitation at low osmolarity opposed the swelling of the muscle cells in the stretched muscles at 37° C while the passive tension *per se* was less important for the degree of swelling. However, had the smooth muscle cells behaved like perfect osmometers the increase in cell water at this reduction in osmolarity would have been 58 per cent. The difference between this value and those obtained in situations where the active tension development was prevented might be accounted for by a loss of intracellular ions. Such a loss of ions was clearly demonstrated in stretched muscles at 37° C which showed the smallest degree of swelling. This suggests that muscle cells, when unable to adapt their intracellular osmolarity to a reduced extracellular tonicity by swelling, do so by dropping a fraction of their intracellular ions.

It was shown in an earlier study concerning the effects of extracellular osmolarity on vascular smooth muscle activity that increases in osmolarity which should make the muscle cells shrink cause inhibition and procedures which should make them swell cause enhancement of their spontaneous activity (Johansson and Jonsson 1968). To be able to understand the mechanism behind this correlation between cell volume and degree of activity it was considered necessary to determine the changes in cell water produced by the variations in extracellular osmolarity. As far as the effect of hyperosmotic solutions is concerned, it has been demonstrated that the intracellular water of the portal vein decreases to an extent that could be expected for a perfect osmometer at increases in osmolarity of the order of 150 mOsm/l (Arvill, Johansson and Jonsson 1969).

Preliminary experiments concerning the effects of hypo-osmotic solutions however, showed that the wet weight of the portal vein did not significantly increase in these media Measurements of extracellular space and total water showed that the results of the wet weight determinations did not merely reflect a redistribution of fluid from extra- to intracellular space, but indicated instead that the swelling of the smooth muscle cells on exposure to hypo osmolarity was restricted in some way One possible explanation for this inability of the muscle cells to swell like osmometers is that some loss of intracellular ions occurs which would still lead to an osmotic balance with the surrounding medium, but at a smaller degree of cellular swelling than would other wise have been required Another possibility would be a restriction of the swelling of the muscle cells due to rigidity of the cell membranes The difference between th osmotic pressures of the intra and extracellular fluids would then be balanced by a greater hydrostatic pressure in the former medium The stimulation of the spon taneous activity in hypo-osmotic solutions and the consequent increase of active ten sion might then contribute to the restricted distensibility of the muscle cells

The aim of the present study was to elucidate the importance of these different factors for the swelling of the muscle cells in hypotonic media Thus experiments were so arranged that active and passive tension of the muscle cells could be inde pendently changed and their influence on cell volume studied Further, the extent to which changes in the concentrations of intracellular ions occur in hypotonic media was estimated in a few experiments by measurements of the muscle contents of potassium

Methods

The experiments were performed on portal vein preparations from rats of the Sprague Dawley strain The body weights of the rats varied between 200 and 250 g

The dissection of the portal vein preparation has been described in detail in a previous article (Axelsson *et al* 1967) though in the present study the vessels were always cut open longitudinally to reduce the diffusion distances and to facilitate the blotting procedure Some of the muscles were mounted on frames that stretched them to their approximate *in situ* length

The changes in cell water produced by different hypo-osmotic test solutions were calculated on the basis of determinations of wet weight sucrose ^{14}C space and total water of the muscles [illegible] wet weight of the muscles were determined during exp[illegible]olutions the muscles were individually mounted on fra[illegible]mes were weighed at intervals on a Cahn electro- balance after a standardized blotting procedure The weight change was expressed in per cent of the mean of three control determinations of the net wet weight of the muscle when exposed to normal Krebs solution for a 30 min period preceding exposure to the hypotonic solution

All muscles used in the sucrose ^{14}C uptake experiments were preincubated for 1/2 hr in normal Krebs solution at 37° C In the experiments where the uptake of sucrose ^{14}C was determined at 1° C the muscles were allowed to accomodate for another 1/2 hr in normal Krebs solution at this low temperature Experiments with recording of mechanical activity showed that 1/2 hr at 1° C was sufficient to abolish all spontaneous activity and nearly all mechanical responses to decreased osmolarity After these preincubation periods the muscles were transferred to the incubation medium which consisted of one of the following solutions normal Krebs (see below) Krebs minus 30 mmoles NaCl/l Krebs minus 60 mmoles NaCl/l or normal Krebs plus noradrenaline 10^{-6} g/ml Labelled sucrose with a specific activity of 35 μc/μmole was added to the incubation media to a concentration of 0 6 mmoles/l The tempera ture of the incubation medium was either 37° C or 1° C After the incubation period the muscles were rapidly washed in non radioactive solution of the same composition as the actual incubation medium blotted between two pieces of filter paper and weighed on a Cahn electro balance The muscles mounted on frames were released before the washing procedure

The preparations were then homogenized in 0 5 ml of 10 % tri-chloro-acetic acid (TCA)
h 10 ml p-dioxane
PO (2 5-diphenyl
and counted in a
standard error of
less than 1 per cent Samples of the incubation media were diluted with 10 % TCA 1 25 before counting The degree of quenching was then found to be the same for the tissue extract
se counting
ie counting
was found

The sucrose space was calculated from the distribution of radioactivity between tissue and medium and is expressed as ml/100 g wet tissue weight

The vessels used when the total water content was to be determined were treated in the same way as the preparations in the sucrose-^{14}C uptake experiments with the only exception that no tracer sucrose was added to the incubation media After determination of the wet weight the muscles were transferred to a vacuum oven and dried to constant weight at 100° C

The normal Krebs solution had the following composition in mmoles/l NaCl 122, KCl 4 73 $CaCl_2$ 2 49, $MgCl_2$ 1 19, $NaHCO_3$ 15 5, KH_2PO_4 1 19 and glucose 11 5 When the effects of noradrenaline were studied, calcium disodium versenate was added to a concentration of 0 026

30 min

The densities of the solutions were measured at 20° C and found to be 1 007 for normal Krebs 1 005 for Krebs minus 30 mmoles NaCl/l, and 1 004 for Krebs minus 60 mmoles NaCl/l

The osmolarity of the normal Krebs solution and of some of the other test solutions was determined by measurements of the freezing point depression Distilled water and a 0 3 M urea solution were used as references to represent 0 mOsm/l and 300 mOsm/l, respectively The mean deviation of several determinations from their average when one and the same sample of normal Krebs

analysed

In a few experiments the potassium contents of portal veins which had first been dried to constant weight in the vacuum oven were determined by ultramicro flame photometry (Haljamäe and Larsson 1968) A more complete investigation of the ionic distribution in the portal vein will be published elsewhere (Haljamae *et al* 1969)

Results

The preliminary experiments referred to above showed that the wet weight of the rat portal vein increased only insignificantly on exposure to hypo-osmolarity The subsequent determinations of the changes in cell water based on measurements of sucrose-^{14}C space and total water contents indicate in accordance with these preliminary results that the cell volume of portal veins is not adjusted to reductions in extracellular osmolarity in such a way as would be expected for perfect osmometers The background of these discrepancies is analysed in the following sections

1 Effects of reduced osmolarity on the volume of the intracellular water in stretched and unstretched portal veins at 37° C

In the present study the changes in total wet weight, produced by the different test procedures were mostly calculated indirectly from the values of dry weights and

sucrose spaces (see Table II) This calculation method is based on the assumption that the weights of the intracellular solids and solutes remain essentially constant when the muscle is exposed to the different test solutions It was found in an earlier

TABLE I

Incubation media	Frames	Sucrose ^{14}C spaces ml/100 g Mean ± S E	n	P_{max}	Dry weights per cent of wet weight Mean ± S E	n	P_{max}	Intra cellular water per cent Mean ± S E	P_{max}
Normal Krebs	+	42.8 ± 0.9	16	0.2	20.7 ± 0.4	7	0.1	100	0.0?
Krebs 60 mmoles NaCl/l	+	41.2 ± 0.9	12		19.6 ± 0.4	6		114 ± 7	
Normal Krebs	−	45.4 ± 1.3	9	0.025	21.0 ± 0.3	13	0.001	100	0.001
Krebs 60 mmoles NaCl/l	−	41.6 ± 0.5	8		18.5 ± 0.4	5		135 ± 9	

study (Arvill Johansson and Jonsson 1969) that such indirectly deduced weight changes were in good agreement with direct measured ones produced by hyper osmotic solutions

Dry weights expressed in per cent of the wet weights and sucrose spaces are given in Table I for muscles incubated in normal Krebs and in Krebs minus 60 mmoles NaCl/l for 15 min at 37° C Some of the muscles were mounted on frames at their *in situ* length and some were unstretched The 15 min value of the sucrose space was

TABLE II Calculation of wet weight (X) of unstretched portal veins incubated for 15 min in Krebs − 60 mmoles NaCl/l at 37 C Weights of extracellular solutes in normal and hypoosmotic Krebs solution were 1.2 g/100 ml and 0.8 g/100 ml respectively

	Normal Krebs	Krebs 60 mmoles NaCl/l
Weight of extracellular solutes g/100 g wet weight	$\frac{1.2 \times 45.4}{100}$	$\frac{0.8 \times 41.6 \times X}{100}$
Weight of total water g/100 g wet weight	79.0	$\frac{81.5 \times X}{100}$
Weight of intracellular solids and solutes	$100 - 79.0 - \frac{1.2 \times 45.4}{100}$	$X - \frac{81.5 \times X}{100} - \frac{0.8 \times 41.6 \times X}{100}$
This value is assumed to be the same in normal and media Thus	$20.5 = X - \frac{81.5 \times X}{100} - \frac{0.8 \times 41.6 \times X}{100}$	
	$X = 113$	

taken to represent extracellular space since it was found in an earlier study (Arvill Johansson and Jonsson 1969) that the uptake of tracer sucrose reached a plateau within that time On the basis of these values the increase of wet weight in the un stretched muscles has been calculated in Table II The wet weight in normal Krebs solution has been arbitrarily set at 100 g By using the respective values of sucrose spaces dry weights and wet weights of the unstretched muscles, incubated in normal Krebs solution and in Krebs minus 60 mmoles NaCl/l, respectively, the intracellular water has been found to increase by 34 per cent during incubation in the latter medium (Table III)

The calculations made in Table II and III can be combined in a single formula

$$\frac{W'}{W} = 100 \frac{\dfrac{1-E}{T-E\,F} - 1}{\dfrac{1-E}{T-EF} - 1}$$

where W stands for the weight of the intracellular water, T for the ratio of dry weight/total wet weight and F for the ratio of weight of extracellular solutes/weight of extracellular space

The symbols with the prime sign refer to muscles incubated in the test solution and the others refer to muscles incubated in normal Krebs solution By inserting all the individual values for sucrose ^{14}C spaces and for dry weights in this formula it is pos-

TABLE III Calculation of increase in intracellular water of unstretched rat portal veins incubated 15 min in Krebs — 60 mmoles NaCl/l at 37° C

	Normal Krebs solution	After 15 min in Krebs solution — 60 mM NaCl/l
Total tissue weight	100	113
Total tissue water g/100 g	79 0	81 5
Total tissue water g	79 0	92 1 (113 × 81 5/100)
Extracellular space ml/100 g	45 4	41 6
Extracellular space ml	45 4	47 0 (113 × 41 6/100)
Spec weight of extracellular fluid	1 007	1 004
Weight of extracellular fluid	45 7 (45 4 × 1 007)	47 2 (47 0 × 1 004)
Weight of extracellular solutes g/100 ml	1 2	0 8
Weight of extracellular solutes g	0 5 (45 4 × 1 2/100	0 4 (47 0 0 8/100)
Weight of extracellular water g	45 2 (45 7 — 0 5)	46 8 (47 2 — 0 4)
Weight of intracellular water g	33 8 (79 0 45 2)	45 3 (92 1 — 46 8)
Per cent increase of intracellular water		34

sible to calculate the mean change of the intracellular fluid volume the standard error of this mean and the statistical significance of the change A computer program was developed and used for performing these extensive calculations The n values applied in these calculations have been the number of observations in the smallest of the 4 series of dry weights and sucrose spaces used in each estimation of a change in the volume of the cell water

The increase in cell water of muscles incubated for 15 min in Krebs minus 60 mmoles NaCl/l at 37° C calculated with the combined formula was 35 ± 9 per cent (n = 5) for the unstretched preparations and 14 ± 7 per cent (n = 7) for those mounted on frames (Table I) All values of the volume of the intracellular water given in the following sections have been calculated with this formula

Had the cells adjusted their volume to the reduced osmolarity like perfect osmotic cells, the intracellular water would have increased by 58 per cent in Krebs minus 60 mmoles NaCl/l (284—179 mOsm/l) It is evident that the increase in cell volume obtained in the present experiments on stretched and unstretched muscles at 37° C were considerably smaller Furthermore, there was a marked difference between the degree of swelling in the stretched and unstretched muscles This discrepancy might reflect a limitation of the swelling of the muscle cells due to the presence of passive tension in the veins of the former group An alternative possibility is that the increase in active tension development caused by the lowered osmolarity would oppose the swelling more forcefully in the stretched muscles where the contractile response should be greater than in the unstretched muscles because of the length—tension relationship (Speden 1960 Sparks and Bohr 1962) These possibilities were examined in the experiments described in Section 2

2 Effects of reduced osmolarity on the volume of the intracellular water in stretched and unstretched portal veins at 1° C

By incubations at 1° C, it is possible to largely eliminate the effects of increased muscle activity on the swelling of the cells produced by hypo-osmolarity, the mechanical responses to lowered tonicity were considerably reduced at that low temperature

Dry weights and sucrose spaces are given in Table IV for stretched and unstretched muscles incubated 15 min at 1° C in Krebs minus 60 mmoles NaCl/l and in Table V for stretched muscles incubated 15 and 60 min respectively in Krebs minus 30 mmoles NaCl/l at the same low temperature Table IV and V also give the corresponding control values obtained at 1° C and the calculated values for the volume of the intracellular water in the different test solutions

The increases in the volume of the cell water of portal veins incubated for 15 min in Krebs minus 60 mmoles NaCl/l were now approximately equal in the stretched and unstretched muscles (40 and 35 per cent respectively) and furthermore of about the same order as in the unstretched muscles incubated in the same solution at 37° C 35 per cent) As shown in Section 1 above the increase in intracellular volume was siderably less (14 per cent) in stretched preparations at 37° C These results

TABLE IV

Incubation media	Frames	Sucrose ^{14}C spaces ml/100 g Mean±S E	n	P_{max}	Dry weights per cent of wet weight Mean±S E	n	P_{max}	Intra cellular water per cent Mean±S E	P_{max}
Normal Krebs	+	40 7±0 7	10	0 001	21 1±0 3	8	0 001	100	0 001
Krebs—60 mmoles NaCl/l	+	34 4±0 9	10		18 5±0 3	8		140±6	
Normal Krebs	—	42 7±0 9	5	0 001	20 9±0 3	7	0 001	100	0 001
Krebs—60 mmoles NaCl/l	—	36 4±0 7	6		18 9±0 2	7		135±6	

indicate that the active tension development obtained in the latter situation may have restricted the swelling of the muscle cells since there was such a pronounced difference between the stretched veins incubated at 37° C and those incubated at 1° C The passive tension *per se* was, however, evidently less important as the increase in cell water was essentially independent of whether frames were used or not in the experiments performed at 1° C

Still the increases in cell water obtained after incubations in Krebs minus 60 mmoles NaCl/l at 1° C were less than would be expected for perfect osmometers (*i e* 58 per cent) When on the other hand stretched veins were incubated for 15 min in Krebs minus 30 mmoles NaCl/l at 1° C, the volume of the cell water increased approximately to the extent expected for such osmotic cells (21 per cent compared to 24 per cent) Furthermore this enlarged cell volume in Krebs minus 30 mmoles NaCl/l remained essentially constant during prolonged exposure to hypo

TABLE V

Incubation media	Time of incubation min	Sucrose ^{14}C spaces ml/100 g Mean±S E	n	P_{max}	Dry weights per cent of wet weight Mean±S E	n	P_{max}	Intra cellular water per cent Mean±S E	P_{max}
Normal Krebs	15	40 7±0 7	10	0 02	21 1±0 3	8	0 01	100	0 001
Krebs—30 mmoles NaCl/l	15	37 6±0 8	14		19 6±0 4	8		121±6	
Normal Krebs	60	44 4±1 0	8	0 02	21 8±0 3	6	0 01	100	0 001
Krebs—30 mmoles NaCl/l	60	41 0±0 2	6		20 1±0 5	6		125±6	

tonicity since the cell water was found to be 25 *per cent* above *control* after 60 min in this media It thus seems as if the muscle cells of *the inactivated portal* vein adjust their volume like osmotic cells *on moderate* reductions in osmolarity while they are unable to do so on more drastic reductions *in the tonicity* of the medium

The calculations of the changes *in the* wet weight and thus also the calculations of the variations in the volume of the cell water are as mentioned above based on the assumption that the weight of the intracellular solids and solutes remains essentially constant during the exposures to the different aniso osmotic solutions In order to check *the reliability* of such indirect determinations of the wet weight *in hypo*-osmotic solutions the total weight changes *were directly measured* in some experiments where stretched muscles were exposed *for 15 min to* Krebs minus 60 mmoles NaCl/l at 1° C These measurements indicated *an increase in* wet weight to 111 per cent ± 1 1 (n = 23) *of the weight in normal* Krebs solution a value in good agreement with the indirectly calculated one of 113 *per cent*

3 Determinations of the potassium contents of portal veins incubated in hypo osmotic solutions

As reported in Section 1 the cell water of portal veins stretched to their in situ length and incubated in Krebs minus 60 mmoles NaCl/l at 37° C increased only by 14 per cent Since this is far less than would be expected for perfect osmotic cells it appears that a great hydrostatic pressure gradient would develop over the cell membranes provided that the muscles maintain their amounts of intracellular ions If on the other hand the muscle cells loose a fraction of their *intracellular ions they* may come to an osmotic equilibrium with the *surrounding medium despite the relatively small increase in cell volume* observed In order to elucidate this possibility the potassium contents of stretched muscles incubated at 37° C in normal Krebs and in Krebs minus 60 mmoles NaCl/l were determined in a few experiments The potassium contents of muscles incubated in normal Krebs was found to be 45 3 ± 0 6 (n = 4) meq K /kg wet weight By using the values for extracellular space (sucrose-^{14}C space) for total water and for extracellular potassium concentration it was possible to calculate the intracellular potassium concentration This would be approximately 117 meq K /kg cell water for muscles in *normal medium* The corresponding figures for veins incubated for 15 min in Krebs minus 60 mmoles NaCl/l at 37° C were 32 8 + 0 5 (n = 4) meq K /kg wet weight and 70 meq K /kg cell water

These values for intracellular potassium ion concentrations have been calculated without correction for the possible existence of bound potassium in the portal vein (cf Haljamae *et al* 1969) but this is probably not important for the present comparisons

Thus the results indicate that the intracellular potassium concentration in the stretched muscles exposed to hypo-osmolarity was considerably reduced compared to control In other words they may still arrive at osmotic equilibrium by losing intracellular ions and the transmembrane hydrostatic gradients may only be transient phenomena

Preliminary experiments indicated that there was also a slight decrease in the intracellular sodium concentration in the stretched muscles exposed to hypo-osmolarity at 37° C which means that the low [K] demonstrated was not due to an exchange of intracellular potassium ions for sodium ions

4 *Effects on cell volume of stimulation with noradrenaline in iso osmotic solutions*
The results mentioned above indicated that the active tension development caused by the lowered osmolarity in stretched muscles at 37° C counteracted the swelling of the cells It therefore seemed to be of interest to investigate whether increased activity of muscles incubated in iso-osmotic medium would influence the cell volume To explore this possibility the changes in intracellular water of stretched and unstretched muscles incubated for 15 min in isotonic Krebs solution plus noradrenaline 10^{-5} g/ml were determined The addition of noradrenaline in this high concentration resulted in a pronounced excitation of the muscle as has been described earlier (Johansson *et al* 1967) It was found that the cell water of the stretched muscles increased to 107 ± 7 per cent (n = 6) and of the unstretched muscles to 111 ± 8 per cent (n = 6) Thus if anything the stimulation of the spontaneous activity caused by noradrenaline gave a slight increase in the cell water though it should be mentioned that the changes were not statistically significant A squeezing out of intracellular water by the increased tension development could thus not be demonstrated in these experiments

Discussion

It was shown in an earlier study (Arvill *et al* 1969) that the cell water of rat portal veins incubated in hyperosmotic solutions decreased to an extent that could be expected for perfect osmotic cells The same type of osmotic behaviour has been reported for guinea pig taenia coli (Brading and Setekleiv 1968) and for frog stomach muscle (Bozler 1965—1966) as well as for striated muscle fibers (Blinks 1965 Dydynska and Wilkie 1963 Bozler 1965—1966) on exposure to hyperosmotic media

The changes in water content of muscle cells incubated in hypo-osmotic solutions seem to be more variable In striated muscle fibers (frog tibial anterior muscle) Blinks (1965) found by using an optical method for cell volume determinations that the cells swelled like perfect osmotic cells on exposure to such solutions On the other hand Bozler (1965—1966) reported that the intracellular water in frog sartorius muscle increased less than expected for osmotic cells on exposure to hypo-osmotic media Bozler's explanation for this difference was that some of the intracellular ions are lost in the hypo-osmotic media and that some of the fiber water is bound and therefore osmotically inactive This author has found in a comparative study that the smooth muscle cells of frog stomach swell to a lesser extent than the cells of the sartorius muscle in hypotonic solutions Similar results with regard to smooth muscle have been reported by Brading and Setekleiv (1968) working on guinea pig taenia coli They found that the swelling of the cells of this musc ...a 25 per cent reduction in osmolarity was less than calculated for a perfect

and they ascribed this to rigidity of the cell membranes Surprisingly, a decrease in the volume of the cell water was obtained by lowering osmolarity to 50 per cent of the normal This was attributed to a damage of the cell membranes in this drastically hypo-osmotic solution

The present determinations of changes in the cell water of vascular smooth muscle incubated in Krebs minus 60 mmoles NaCl/l (105 mOsm/l below normal) show that the muscle cells are unable to adjust their volume like perfect osmometers at this degree of hypo osmolarity although the extent to which they swell varies with the experimental conditions The results indicate that the increased active tension development induced by the lowered osmolarity counteracts the swelling of the muscle cells When the contractile response to hypo osmolarity was hampered by a low incubation temperature the muscle cells swelled almost like perfect osmometers on a reduction in the osmolarity by 55 mOsm/l A similar effect of the contractile response on the swelling of the cells of frog stomach muscle in solutions with high KCl concentration was demonstrated by Bozler (1962)

The muscle cells of the unstretched veins or of veins incubated at 1° C swelled considerably more than the cells of stretched muscles incubated at 37° C in Krebs minus 60 mmoles NaCl/l but the swelling was always less than expected for perfect osmometers This could indicate either that the cell membranes become rigid in conjunction with a more pronounced swelling of the cells An alternative explanation is that an active tension response to lowered osmolarity is still maintained at 1° C and in the absence of passive tension and that this response to some extent interferes with the cellular swelling

That the discrepancy between the measured and the theoretically calculated increase of cell water in Krebs minus 60 mmoles NaCl/l should be due to the existence of a bound fraction of cell water is contradicted by the fact that the muscle cells behave almost like perfect osmometers in hyperosmotic media and in Krebs minus 30 mmoles NaCl/l at 1° C

Although the active tension development in the stretched muscles exposed to Krebs minus 60 mmoles NaCl/l at 37° C seems to have counteracted the swelling of the muscle cells it is unlikely that the contractile response would cause a maintained increase in the intracellular hydrostatic pressure As it was found that these muscles lose intracellular ions there are reasons to believe that a transmembrane osmotic equilibrium is restored despite the restricted swelling A similar loss of ions in hypo-osmotic media has been reported for frog stomach muscle (Bozler 1965–1966) and for guinea pig taenia coli (Brading and Setekleiv 1968) Preliminary experiments have suggested that even unstretched veins incubated at 1° C in Krebs minus 60 mmoles NaCl/l lose ions in spite of the fact that they swell considerably more than the stretched muscles at 37° C This suggests that the slightly restricted swelling of these essentially inactive muscles is compensated osmotically by an adjustment of their intracellular ion concentrations

The effects of noradrenaline on cell water in iso-osmotic solutions indicate that a marked increase in active tension does not generate intracellular hydrostatic pressures

of such a magnitude as to cause 'filtration' of fluid out from the cells However, as mentioned above the contractile response to lowered osmolarity seems to play a role in opposing the swelling of the muscle cells

The experiments performed in the present study show in summary that the cells of vascular smooth muscle at 37° C swell at least to some extent in hypotonic solutions The changes in the transmembrane concentration gradients of ions associated with the variations in the cell volume may partly explain the changes in the electrical and mechanical activity of the muscle described in a concomitant article (Jonsson 1969) In our previous studies concerning the effects of hyperosmolarity, it has been discussed whether changes in the permeabilities of the cell membranes to ions may contribute to the adjustments in membrane potential obtained by variations in the osmolarity (Johansson and Jonsson 1968) These problems will be subjected to further analysis by studying the wash-out of ^{42}K and ^{24}Na from portal veins in solutions of different osmotic strengths

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Local Transmitter Concentrations in Vascular Smooth Muscle during Vasoconstrictor Nerve Activity

By

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Abstract

LJUNG B *Local transmitter concentrations in vascular smooth muscle during vasoconstrictor nerve activity* Acta physiol scand 1969 77 212—223

Adrenergic excitatory responses of a nerve muscle preparation consisting of the rat portal vein and its sympathetic post ganglionic nerve supply were studied quantitatively *in vitro* The maximal contractile response to nerve stimulation was obtained at 16 imp/sec and it averaged 56 per cent of the maximal response to exogenous noradrenaline NA (10^{-4} M)

Factors such as the myogenic propagation between the individual muscle cells and the sparse distribution of the sympathetic nerve fibres do not allow an estimation of the transmitter concentration during nerve activity from a mere comparison of the magnitude of the responses to nerve stimulation and to exogenous NA respectively It was shown however that the degree of reduction of the contractions induced by exogenous NA after partial α receptor blockade by phenoxybenzamine was inversely related to the concentration of NA in the bath By comparing the reductions of the neural responses with the reductions of the responses to graded concentrations of exogenous NA the effective transmitter concentration could be estimated The values obtained were $(8 \pm 4) \times 10^{-6}$ M at 4 imp/sec and $(2 \pm 0.5) \times 10^{-5}$ M at 16 imp/sec

The effectiveness of α adrenergic blocking agents in affecting neural and humoral responses to catecholamines *in vivo* is discussed in the light of the present findings

Recent development in noradrenaline (NA) assay methods and refined techniques for morphological and functional studies on the peripheral adrenergic system have led to a rapid increase in the understanding of sympathetic control mechanisms (for ref see *e g* Iversen 1967) Studies on the vasoconstrictor fibre system have provided quantitative information concerning the extent of transmitter release and the relative importance of different local transmitter eliminating mechanisms (Folkow Haggendal and Lisander 1968) It is evident that the amount of NA reaching the adrenergic smooth muscle receptors will be dependent on the quantity released per impuls the impulse frequency and the rate of transmitter elimination but factors such as the degree of dilution in the neuromuscular gap also come into play Obviously it would be difficult to quantitate the influence of each of these processes for calculation of the transmitter concentration during vasoconstrictor fibre activation at dif

ferent impulse rates In the present study an attempt was made to estimate average effective concentrations of transmitter in the neuromuscular gaps of vascular smooth muscle by analysing the α receptor stimulating effect of NA liberated during nerve activity

The experiments were carried out *in vitro* using isolated nerve muscle preparations of the rat portal vein The sympahetic innervation of this vessel is distributed in sparse two dimensional plexus so that in fact only a fraction of the muscle cells are 'directly innervated (Malmfors *et al* to be published) Due to myogenic propagation the nerve responses become augmented as 'non innervated cells are recruited (Johansson and Ljung 1968) Consequently responses to sympathetic stimulation and to exogenous NA, respectively reflect two different means of excitation A direct comparison of their magnitudes would therefore not yield relevant information for estimation of effective transmitter concentrations

It was shown however, that the *relative reduction* of a response to exogenous NA after partial α receptor blockade with phenoxybenzamine, was dependent on the agonist concentration By comparing quantitatively the relative reductions of responses to graded concentrations of exogenous NA with the reductions of neural responses after partial α receptor blockade an approximate estimation was obtained of the NA concentrations which act on the α receptors during sympathetic nerve stimulation

Part of the present results has briefly been reported at the Scandinavian Physiology Meeting in Goteborg March 1968 (Ljung 1968)

Methods

The results of this report were obtained in 44 expts in which a nerve muscle preparation [illegible]

by cutting the retroperitoneal tissue One end of the portal vein was attached to a muscle holder in a 30 ml mantled organ bath The activity of the longitudinal smooth muscle of the vessel was recorded under isometric conditions by attaching the other end to a force displacement transducer (Grass FT 03) operating a direct writing oscillograph (Grass polygraph) [illegible]

[illegible] fore the experiment was started The preparation was exposed to propanolol 10^{-6} M every 60 min to maintain a *continuous β receptor* blockade

The contractile responses were quantitated by means of an electronic integrating device giving deflections proportional to the area per unit time between the baseline of passive tension and the active tension curve Each experimental response was calculated as the area recorded during the influence of nerve stimulation or injected NA minus the average area recorded during a comparable preceding period of spontaneous activity

The modified Krebs solution used in all experiments had the following composition in mM NaCl 122 KCl 4 73 $CaCl_2$ 2 49 $MgCl_2$ 1 19 $NaHCO_3$ 15 5 KH_2PO_4 1 19 CaNa-versenate 0 026 and glucose 11 5

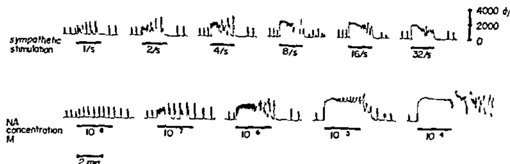


Fig 1 Spontaneous activity and adrenergic excitatory responses of the rat portal vein, recorded as isometric tension *in vitro*

Upper recordings Postganglionic sympathetic stimulation with supramaximal intensity, graded impulse frequencies

Lower recordings Graded concentrations of exogenously administered NA

It was continuously bubbled with 4 % CO_2 in O_2 The temperature was kept constant 38° C

The drugs used were

l noradrenaline bitartrate (Nor Exadrin® Astra) phenoxybenzamine HCl (Dibenzyline Smith, Kline and French) and propranolol HCl. Drug concentrations were expressed in M. A stock solution of propranolol was made by dissolving the substance in saline to a concentration of 10^{-3} M The other drugs were obtained in commercial concentrated solutions The stock solutions were diluted with saline immediately prior to the injection into the bath

Results

1 *Quantitation of adrenergic excitatory responses*

Typical recordings of spontaneous activity and adrenergic excitatory responses are illustrated in Fig 1 The contractions of the rat portal vein induced by graded postganglionic sympathetic nerve stimulation (upper row in Fig 1) resemble in all respects those previously described for a similar preparation from rabbit (Johansson and Ljung 1967 a) The responses to increasing concentrations of exogenous noradrenaline (NA) are seen in the lower part of Fig 1

The contractile responses have been quantitated and expressed in per cent of the responses to NA 10^{-4} M in each experiment Data from 10 expts are represented in Fig 2 where the mean response ± S E M has been plotted against log NA concentration and log impulse frequency respectively The dose-response relationship displays a characteristic S-shape showing a maximal effect of NA 10^{-4} M At still higher concentrations there was no further increase in tension

The frequency response curve is flatter with maximum tension developed at 16 imp/sec As seen in the diagram maximal response to sympathetic nerve stimulation cannot develop the full tension obtainable by adding NA into the bath In 40 expts the mean tension developed at 16 imp/sec amounted to 56 ± 5 per cent (± S E M) of the corresponding responses to NA 10^{-4} M

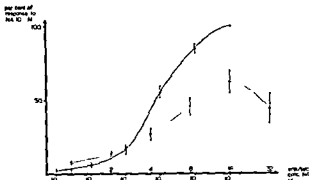
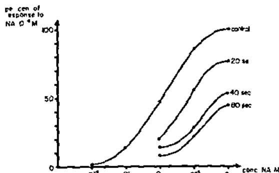


Fig 2 Excitatory responses of rat portal vein to sympathetic stimulation (dotted line) and to graded concentrations of exogenous NA (full line), expressed as a per cent of responses to NA 10^{-5} Mean ± S E M from 10 expts

2 Characteristics of partial a receptor blockade

Phenoxybenzamine in concentrations around 10^{-8} M and higher completely abolished all adrenergic excitatory responses in most experiments With lower concentrations a partial blockade developed This is illustrated in Fig 3 where the mean responses of two nerve muscle preparations have been plotted to show the effect of increasing the time of exposure to the *a* blocker A gradual development of the blockade is seen with increasing durations of exposure It is characterized by a shift of the response curves to the right which implies that higher impulse frequencies and higher NA concentrations respectively are now required to elicit a given re



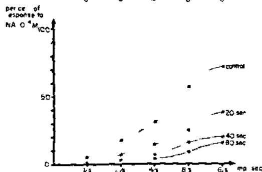


Fig 3 Rat portal vein *in vitro* Partial blockade of responses to graded concentrations of exogenous NA (full line above) and to sympathetic stimulation (dotted line below) Preparation repeatedly exposed to phenoxybenzamine 10^{-10} over increasing periods of time Note the rapid onset of the blockade of both neural exogenous NA responses Curves connecting measured values were drawn to illustrate the principle of non equilibrium antagonism Mean of 2 expts

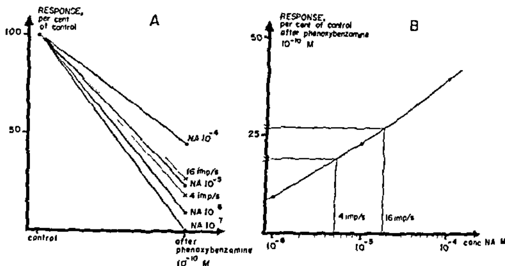


Fig 4 *Panel A* Results of one experiment on rat portal vein with partial α receptor blockade by phenoxybenzamine 10^{-10} *Responses remaining after blockade are expressed as a per cent* of control values Note that the reductions of responses to exogenous NA are inversely related to agonist concentration Neural responses are more reduced than the response to NA 10^{-4} but less than that to 10^{-6} *Panel B* Calculation of transmitter concentration in the experiment of panel A For explanation see text

sponse However, the peak responses are also lowered so that maximal contractions developed before the partial blockade, are no longer obtainable, even if the concentration of the stimulatory agent is further increased After the blocking agent had been rinsed out from the bath, the degree of blockade remained stable for the rest of the experiment in spite of repeated periods of sympathetic stimulation and exposures to NA These latter features reflect the "non-equilibrium" antagonism exerted by phenoxybenzamine (Nickerson 1959)

In the present study it was important to obtain uniform penetration of the blocking agent into the entire vascular wall The time course for the onset of the blockade was found to be very rapid in the portal vein preparation as seen in Fig 3 and the exposure period of 3 min, chosen for the subsequent experiments, was sufficient for the α-blockade to develop uniformly in the vessel

The degree of partial α receptor blockade was varied by using phenoxybenzamine concentrations between 10^{-11} and 3×10^{-10} M With higher concentrations the blockade became too pronounced, leading to very small contractions These would not allow an accurate quantitation since variations in the spontaneous activity considerably influenced the calculation of the net excitatory response

The pattern of reduction of the adrenergic excitatory responses is illustrated in Fig 4A which summarizes the results of 1 expt The responses to graded concentrations of exogenous NA were compared quantitatively before and after partial α-receptor blockade (phenoxybenzamine 10^{-10} M) The same was done for the nses to nerve stimulation The responses to the different concentrations of NA ned after phenoxybenzamine were expressed as a per cent of the responses to

the same amounts of NA obtained before the α receptor blockade. The effect of NA 10^{-7} M was completely abolished whereas NA 10^{-4} M still gave a response which was 43 % of the control. The corresponding figure for NA 10^{-6} M was 9 % and for NA 10^{-5} M 23 %. The order of reduction of the responses to NA in the medium caused by the partial blockade is thus inversely related to the NA concentration. This pattern was consistently seen in each of the 35 expts where such comparisons were made independent of the degre of partial α receptor blockade. When in such experiments the preparation was exposed to a second slightly higher concentration of phenoxybenzamine the responses were further reduced and, whether expressed as a per cent either of initial control values or of the responses after the first partial α receptor blockade the same distribution of the remaining responses was obtained. The effect of 10^{-6} M was reduced to the greatest extent that of 10^{-4} M to the least.

In Fig 4A also the responses to sympathetic nerve stimulation after the partial blockade have been expressed in per cent of the corresponding control values. It can be seen that their relative reductions were less pronounced than those found for the highest concentrations of exogenous NA. The response to 16 imp/sec was less affected than that to 4 imp/sec.

3. *Use of partial α receptor blockade for estimation of transmitter agent concentration*

Provided that the degree of reduction of the adrenergic responses caused by a certain amount of phenoxybenzamine is primarily dependent on the effective concentration of NA acting on the α receptors the data given in Fig 4A would permit an estimation of the concentration of NA which stimulates the repectors during sympathetic nerve activation at 4 and 16 imp/sec. This estimation would be based on a comparison of the reduction of the neural responses after the partial α blockade in relation to the distribution of the decreased responses to the different NA concentrations. The method of making this comparison is examplified in Fig 4B. The per cent responses remaining after the partial blockade have been plotted against log concentration of exogenous NA. By interpolation the concentrations of the transmitter acting on the receptors during nerve stimulation at 4 and 16 imp/sec were obtained from this curve after inserting the relative reductions of the neural responses on the ordinate.

This procedure was used for an estimation of transmitter concentration in 15 expts.

The concentrations obtained amounted to $(8 + 4) \times 10^{-6}$ M at 4 imp/sec and $(2 \pm 0.5) \times 10^{-5}$ M at 16 imp/sec (mean $\pm$ S.E.M.).

It is essential for the validity of the present approach to the determination of transmitter concentrations at the neuro muscular gaps that the α receptors stimulated by neurogenic and by exogenous NA respectively are eqaully accessible to the blocking agent. The use of low concentrations of phenoxybenzamine over short exposure periods makes this question particularly important. In order to elucidate the accessibility of the sets of adrenergic α receptors stimulated by externally ad-

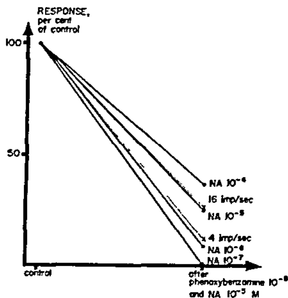


Fig 5 Rat portal vein Partial α receptor blockade by phenoxybenzamine 10^{-8} obtained during receptor protection" through the competitive influence of NA 10^{-5} Note the same pattern of distribution of remaining responses as in Fig 4A

ministered NA and by sympathetic nerve activity respectively 5 expts were performed in which a partial α receptor blockade was obtained with comparatively high doses of phenoxybenzamine and simultaneous 'receptor protection (Furchgott 1954) through the competitive action of NA present in the bath (Nickerson and Gump 1949, Seed and McKay 1949) One such experiment is illustrated in Fig 5 A series of control responses were first established The preparation was then exposed to a high concentration of NA (10^{-5}) and when the excitatory response was fully developed phenoxybenzamine 10^{-8} M, was also added to the Krebs solution for 5 min with NA still present in the bath This concentration of the blocking agent would have extinguished all adrenergic responses in most experiments but as the α receptors were now protected by competition between agonist and antagonist only a partial blockade prevailed after the two agents were rinsed out All subsequent responses again expressed as per cent of the initial ones were distributed in exactly the same order as in the foregoing experiments with lower doses of phenoxybenzamine As seen in Fig 5 the reductions of the responses to nerve stimulation fall in positions comparable to those in Fig 4A The partial phenoxybenzamine blockade obtained in these experiments as a result of competition between NA and the blocking agent thus affected adrenergic responses of the preparation in the same way as when the partial blockade was produced by low doses of phenoxybenzamine and shorter exposure periods

Discussion

The nerve muscle preparation of the portal vein is composed of myogenically active smooth muscle of the propagating type supplied with an excitatory sympathetic innervation (Johansson and Ljung 1967 a b This *in vitro* preparation was used

in the present study in an attempt to elucidate certain quantitative aspects of neuromuscular function in vascular smooth muscle

Three major problems were investigated during these experiments First the excitatory frequency response and dose response relationships to sympathetic nerve stimulation and to exogenously administered NA respectively Second the relative effectiveness of phenoxybenzamine in blocking responses to neural stimulation and responses to graded concentrations of exogenous NA Third the mean effective transmitter concentration during nerve stimulation at 4 and 16 imp/sec The results of the investigations of the first two problems were utilized in the study of the third

1 *Quantitation of excitatory adrenergic responses*

It would be difficult to predict the relative magnitude of the contractile responses obtained by neural stimulation and by exogenous NA respectively Nerve stimulation would be expected to exert a relatively weaker influence due to a rather sparse innervation of the portal vein demonstrated by histochemical studies (Malmfors *et al* to be published) The neurogenically induced activity in the directly innervated muscle cells is however propagated by myogenic mechanisms to adjacent cells so that the neural responses become augmented to some extent (Johansson and Ljung 1968)

Besides the paucity of the nerve supply there are several reasons why nerve stimulation should give weaker responses When the muscle is exposed to exogenous NA the positive inotropic effect of NA (Johansson *et al* 1967) may contribute to the enhanced tension development of the entire muscle but the force of contraction should not be increased by this mechanism in cells which are activated indirectly by myogenic spread of excitation from innervated foci Another factor which could limit the neural responses is damage to nerve fibres during dissection and in the course of the experiment

The maximal response to sympathetic stimulation at 16 imp/sec averaged 56 per cent of that to NA at 10^{-4} M

In view of the sparse innervation the myogenic propagation and the preparatory trauma direct comparisons of the magnitude of responses to sympathetic nerve stimulation and to exogenously administered NA would not be relevant for an estimation of the transmitter concentrations which act on the adrenergic receptors during nerve activity

2 *Relative potency of phenoxybenzamine in blocking responses to neural and exogenous NA*

In the present study it was shown that the relative reduction of the contractile responses of the portal vein after a certain exposure to phenoxybenzamine was inversely related to the concentration of the agonist exogenous NA (Fig 4A) If it is assumed that all *α* receptors of the preparation were uniformly blocked by phenoxybenzamine one would expect the responses to injected NA to be reduced to a greater extent than the neural responses as long as the concentration of administered NA is below that obtained in the neuro-muscular gap during neural activation On the

other hand the stimulatory effect of higher concentrations of NA would be relatively less impaired As seen in Fig 4A the reductions of the neural responses were in fact less pronounced than the reductions of the responses to low and moderate amounts of exogenous NA and the effects of the highest NA concentrations were better maintained than the neural responses

The diagram shown in Fig 4A may be considered to represent graphically the relation between the remaining response and the effective local agonist concentration allowing a comparison between the *reductions* of responses to neural and to exogenously added NA

A quantitative evaluation of the effectiveness of phenoxybenzamine in blocking responses to sympathetic nerve stimulation and exogenously administered NA respectively would be misleading unless the effective local concentrations of NA were taken into account As pointed out by Trendelenburg (1963) experimental quantitation of reductions of responses to a certain agonist after administration of antagonists are of very limited value unless due respect is paid to the agonist concentration and the dose response relationships This must be equally important when comparisons are made between the effect of an antagonist on the responses to an agonist when the latter is released from nervous structures and when it is exogenously administered respectively

The local NA concentration existing during sympathetic nerve activation greatly exceeds that in arterial blood during even intense catecholamine release from the adrenal medulla (see further below) and it also exceeds the concentrations which for practical reasons can be obtained by ia or iv administration *in vivo* Therefore phenoxybenzamine and related substances when studied *in vivo* will reduce adrenergic neural responses to a smaller extent than those induced by exogenous NA (*e g* Youmans *et al* 1955 Schmitt 1957 Henning and Johnsson 1967) This has led to a general concept of the *α* receptor blocking agents as more readily antagonizing responses to circulating NA than to nerve stimulation (*cf* Goodman and Gillman 1965 p 549)

The reverse has also been reported Levin and Beck (1967) found a more pronounced reduction of neural constriction in the hindlimb of the dog after administration of phenoxybenzamine As discussed by these authors the presence of a sympathetic dilator system like the cholinergic fibres in skeletal muscle may explain this phenomenon With increasing adrenergic *α* blockade the vasodilating influence could be unmasked and the neurogenically induced vasoconstriction relatively more reduced

3 *Estimation of mean effective transmitter concentration*

The relationship shown between agonist concentration and reduction of effector response after a partial receptor blockade was utilized in the estimation of transm t er concentration It was then necessary to make some assumptions regard ng the perties of the transmitter the receptors and the distribution of exogenou ly ad istered substances

It was previously demonstrated (Johansson and Ljung 1967 a) that the neural responses of the nerve muscle preparation *in vitro* were eliminated by adrenergic blocking agents It is therefore assumed that the transmitter agent in its actions is entirely comparable to injected l NA Further, it has to be assumed that endogenous and exogenous NA stimulate receptors that possess the same characteristics regarding affinity for the agonist and subsequent intrinsic activity of the drug receptor complex

As to the possible existence of inhibitory nerve fibres no evidence has been found for this under the present experimental conditions, since there was no clear-cut reduction in spontanous activity in response to nerve stimulation after α receptor blockade This also indicates that the β stimulating action of the endogenously released NA is not capable of inducing β adrenergic inhibition of portal vein activity Exogenously administered NA can, however, exert a β stimulatory action on the portal vein after phenoxybenzamine (Johansson *et al* 1967) Consequently it was important to secure complete β receptor blockade for the present quantitation of the α adrenergic blockade This was accomplished without obvious disturbances from the potential local anesthetic effect of propranolol

In studies utilizing *in vitro* techniques the concentrations of the administered drugs are as a rule considered equal at all the receptors throughout the preparation In many tissues this assumption may not be justified as pointed out for instance by Nickerson (1967) Diffusion barriers may considerably hamper the drug equilibration and local transmitter eliminating mechanisms may cause a reduction of the concentration of the agonist, especially close to nerve terminals In the thin portal vein, however, diffusion of substances of moderate molecular size, such as sucrose evidently occurs quite rapidly (Arvill Johansson and Jonsson 1969) Such rapid equilibration is probably reflected also in the very immediate onset of responses to agents like NA when added to the bathing solution (see Fig 1) Evidently the drugs are also distributed uniformly within the muscle layers as indicated by the fact that the relative blockade of the neural responses and of those to exogenous NA obtained by low concentrations of phenoxybenzamine is as effective after 20 sec as it is after a total period of exposure of 140 sec (Fig 3) or more

In the experiments where a partial blockade was obtained by a relatively high concentration of phenoxybenzamine maintained for 5 min while the α receptors were being 'protected' by a simultaneous exposure to NA (Fig 5) the reductions of the responses displayed the same pattern as in Fig 4A where only a low dose of phenoxybenzamine had been administered This indicates that the concentration of the drugs and the accessibility of the entire α receptor population for both NA and phenoxybenzamine are reasonably uniform

The receptor protection experiments also demonstrate that the potential ability of phenoxybenzamine to antagonize acetylcholine histamine and 5 hydroxytryptamine (Graham 1962) did not influence the study as the presence of NA during the development of the blockade is known to protect the α receptors selectively (Furchgott 1954)

The dominating mechanism for elimination of NA, released during vasoconstrictor fibre activation, is active re-uptake of the agent into the adrenergic neuron (*cf* Iversen 1967, Folkow, Haggendal and Lisander 1968) The uptake of labelled NA into nervous structures of the portal vein was not reduced after exposure phenoxybenzamine 10^{-9} M for 10 min (Jonasson and Ljung unpublished observ tion) Thus the very low concentrations of the blocking agent, used in this stud did not affect the concentration of NA in the neuro muscular gap

It is concluded that the present approach to the assessment of transmitter concer tration provides reasonably reliable information about the level of NA which ac on the receptors during neural activation The values obtained, $(8 \pm 4) \times 10^{-9}$ M a 4 imp/sec and $(2 \pm 0.5) \times 10^{-5}$ M at 16 imp/sec, represent the mean local concer trations acting on the receptors of the smooth muscle cells at the respective impul frequencies

Uptake of exogenous NA by the nerve endings might affect the results in such way that the curve of Fig 4B would be displaced to the left In fact, a moderat increase in the responsiveness of the portal vein to exogenous NA has been observe after cocaine treatment and chronic denervation (Johansson and Ljung unpublishe observation)

In a recent study Folkow Haggendal and Lisander (1968) calculated the NA concentration in the junction gap at vasoconstrictor nerve endings in the calf muscl of cat during sympathetic stimulation Their data were based on assessments of the amount of NA appearing in the venous outflow after blockade of the local eliminating mechanisms From histological data concerning the number of adren ergic varicosities per unit tissue weight the amount of NA released per impul e from each varicosity and the resulting concentration in the neuro effector junction gap was calculated The latter figure around 10^{-6} M is of the same order of magni tude as the present results

It is evident that the local transmitter concentration during vasomotor fibre ac tivation with physiological impulse frequencies (Folkow 1952) is about 1000 times higher than the concentration of NA in the circulating blood (*cf* Folkow Haggen dal and Lisander 1968) This has to be taken into consideration in physiological and pharmacological studies on the sympathico adrenal system

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Drugs were generously [illegible] ® conc) and by AB Melo (Dibenzyline®)

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Hyperosmolality and Vasodilatation in Human Skeletal Muscle

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Abstract

LUNDVALL, J, S MELLANDER and T WHITE *Hyperosmolality and vasodilatation in human skeletal muscle* Acta physiol scand 1969 77. 224—233

Exercise of the forearm muscles in *man* was *found* to cause a considerable increase of the osmolality in the venous effluent but no osmolar change in arterial blood, thus indicating a regional hyperosmolality in the working muscles There was a relation between the degree of *hyperosmolality during work and the extent of the exercise hyperemia* (determined immediately after cessat [illegible] produced in the resting [illegible] cose, xylose or mannito [illegible] ere was no significant [illegible] essively with increasing osmolality Furthermore, intramuscular deposits of Xe^{133} in the resting muscle were cleared more rapidly when the tracer was dissolved in hypertonic than in isotonic medium indicating a hyperemia response also when the hyperosmolality primarily involved the extra vascular space [illegible] osmolality on sk [illegible] muscle *in vitro* [illegible] to exercise hyperemia in man

Functional hyperemia in exercising skeletal muscle is one of the most drastic vascular adjustments occurring in the organism This may be illustrated by the fact that in the adult human, blood flow to the skeletal muscles can increase from a value of about 0 8 l/min at rest to about 15 to 20 l/min during strenuous exercise It is generally *agreed that the regional vascular response, the* vasodilatation in muscle is caused to the greatest extent by some local chemical metabolic factor the nature of which however, is still disputed (for details see Barcroft 1963, Mellander and Johansson 1968) It appears at present most likely that several chemical factors are involved in the reaction Tissue hypoxia and an increased potassium concentration in the interstitial space of muscle seem to play important roles in the exercise hyperemia response, at least in anesthetized experimental animals (Kjellmer 1964 Skinner and Powell 1967), but these two factors can apparently not fully explain the phenomenon (Mellander and Johansson 1968)

Recently, experimental evidence was presented to suggest that a locally produced

increase of osmolality in the exercising muscle is a factor that can contribute significantly to the development of the functional hyperemia response (Mellander *et al* 1967, Gray *et al* 1968) Those experiments were performed on a calf muscle preparation in anesthetized cats and also on isolated vascular smooth muscle *in vitro* Exercise of the calf muscles was associated with a considerable increase in muscle osmolality as reflected in the venous effluent A relation existed between the degree of hyperosmolality and of exercise hyperemia Experimental hyperosmolality of similar magnitude as that present in exercise, produced in the resting skeletal muscle by intra arterial infusions of various hypertonic solutions evoked a pronounced dilatation of the resistance vessels especially in the high osmolality range Furthermore the pattern of response of the precapillary sphincters and the capacitance vessels resembled that observed during work

The mechanisms by which hyperosmolality inhibits vascular smooth muscle tone were elucidated by studying the electrical and mechanical activity in the isolated spontaneously contracting rat portal vein Increased osmolality caused pronounced and sustained relaxation mainly by inhibiting myogenic pacemaker activity, an effect ascribed to changes in transmembrane ionic concentration gradients and in membrane permeabilities to ions, the latter change caused by osmotic reduction of smooth muscle cell volume (see also Johansson and Jonsson 1968)

The hyperosmolality in muscle that occurs in exercise *in vivo* is apparently caused by release of products from the contracting striated muscle cells primarily into the interstitial space and this alteration in the environment of the vascular smooth muscle might decrease vascular tone by the mechanisms discussed

Although there is evidence to indicate that hypoxia increased potassium concentration, and hyperosmolality are important local chemical factors contributing to exercise hyperemia elicited during somatomotor fibre stimulation of the skeletal muscles in anesthetized animals the mechanisms responsible for exercise vasodilatation in man are still largely unknown In the present study an attempt was made to investigate whether the osmolar factor could be involved in this reaction in conscious man in response to voluntary exercise The approach was basically similar to that used in the experiments on cat calf muscle mentioned above Regional osmolar changes and alterations in blood flow were studied during graded forearm exercise These effects were compared to the vascular reactions in the resting forearm in response to experimental hyperosmolality produced by intra arterial infusions of hypertonic solutions Furthermore by the use of the Xe^{133} clearance method flow rates were compared when the tracer—deposited in the extravascular space of muscle—was dissolved in hypertonic and isotonic medium respectively A brief preliminary report of this study was presented elsewhere (Gray *et al* 1968

Methods

Observations were made on 22 healthy males 22 to 47 years old mean 30 years Circulatory and osmolar changes were studied in the forearm in 18 subjects (1 n response to exercise and 13 in response to hypertonic infusion *i e* 6 subjects were exposed to both these experimental procedures) Clearance studies of Xe^{133} were performed on 4 subjects

In the studies on the forearm, a polyethene catheter was inserted percutaneously into the left brachial artery in the antecubital fossa, as described by Berneus *et al* 1954, and advanced about 12 *cm* in the proximal direction This catheter was used for infusions of hypertonic or isotonic solutions and for the sampling of blood for osmolality determinations Venous blood from the left forearm was sampled from a polyethene catheter inserted *via* a suitable antecubital vein into a deep distal vein, if this was technically impossible, it was inserted in the proximal direction into a deep cognate vein In some experiments two venous catheters were used, one in each direction Care was taken that the tip of the proximal venous catheter was always a few cms distal to the tip of the arterial catheter to avoid admixture of blood from regions not exposed to the intra-arterial infusion The distal venous catheter was directed towards the flexor muscles of the forearm

Forearm blood flow was measured by venous occlusion plethysmography using a water filled temperature-regulated (34° C) plethysmograph similar to that described by Dahn (1964) The subjects were placed comfortably in recumbent position Flow measurements were usually made on both forearms simultaneously During blood flow measurement, and during sampling of blood, hand circulation was occluded by inflating cuffs around the wrists to a pressure about 50 mm Hg above the systolic pressure

Arterial blood pressure was measured in the right arm by auscultation Mean arterial blood pressure was taken as the diastolic pressure plus one third of the pulse pressure

The subjects exercised the flexor muscles of the forearm by compressing a spring-loaded hand ergometer between the fingers and the palm of the hand The subjects were instructed to perform each compression as rapidly as possible and then relax the fingers completely until the next compression The intensity of the exercise was varied by altering the frequency of the hand contractions within a range from 1/8 sec up to 1/sec It was not possible to quantitate the work load in absolute units by this technique, but, in one and the same subject, exercise hyperemia responses could be elicited which were related to the frequency of hand contraction

Isotonic and hypertonic solutions were infused into the brachial artery at rates varying from 0 95 to 3 0 ml/min (except for one case when it was 5 3 ml/min) using a constant infusion apparatus In most cases, solutions of glucose (5 5, 10, and 20 %, w/v) were given, but mannitol (5 and 15 %), and xylose (4 2 and 20 %) were also tested The solutions were sterile and pyrogen-free, and no untoward effects of the infusions were observed The infusions were administered after the forearm had been at rest for at least 20 min

... osmolality were withdrawn from the arterial and ... ollected ... nolality ... fug ng and the measurement of osmolality was made within 1 h after sampling Plasma osmolality was measured by thermistor cryoscopy (Osmometer 31 LAS Advanced Instruments, Inc) and each sample was measured twice If occasionally different readings were obtained the mean value was used Spread of readings in repetitive measurements on osmolar standards was at most ± 1 5 mOsm/kg

The experimental procedures in the studies on the forearm were as follows Blood flow and arterial blood pressure were measured repetitively and several blood samples for osmolality ... at least 20 min ... after cessation of the exercise) In most cases, exercise was performed at a ... the frequency of hand contractions and it was interrupted only during the periods of flow measurement

... hypertonic solutions were given to the resting forearm after the ... were ... minations of pO_2 ... and Bradley 1958) ... ion

In the experimen ... ... were studied blood flow in the anterior tibial muscle was estimated by the X... clearance method as described by Lassen *et al* 1965) In each experiment, simultaneous clearance studies were performed on both legs The Xe^{133} solution (0 1 to 0 2 ml) was injected into the right and left anterior tibial muscles at approximately the same site and depth. One side received the Xe^{133} as supplied by the manufacturer (The Radiochemical Centre,

Amersham, England), i e 1 mc/ml in 0 9 % sodium chloride solution ("isotonic solution) The opposite side received the Xe^{133} solution mixed with a sufficient volume of a hypertonic solution of glucose or mannitol to give desired increases in osmolality of the injected solution The measurements were made under resting conditions in the supine position

Results

The present experiments show that exercise in man is associated with an increased osmolality in the working skeletal muscles as reflected in the venous effluent During work performed with the flexor muscles of the forearm, plasma osmolality in venous blood from the region invariably rose above the control level at rest (maximum increase observed being 27 mOsm/kg), whereas arterial plasma osmolality was not significantly altered Venous plasma osmolality increased quite rapidly after the onset of forearm exercise and appeared to reach a maximum level within about 2 to 3 min During more prolonged periods of exercise at a given rate of hand contraction there was, as a rule, only moderate deviations from this level of hyper osmolality After cessation of exercise, venous osmolality declined rapidly and returned to the control level within 2 to 3 min The osmolality values at each level of forearm exercise given below refer to samples taken about 2 to 3 min after the onset of work

In the same experiment, there was a relationship between the frequency of hand contraction and the extent to which venous osmolality rose above the resting control value There was also a relationship between the extent of hyperosmolality in exer cicse and the magnitude of the hyperemia in the immediate post-contraction period For example, one subject with a resting flow of 2 2 ml/min × 100 ml tissue showed, at stepwise increases of forearm exercise, a rise in venous osmolality of 6, 8, 10, 13 and 15 mOsm/kg and corresponding blood flow values of 3 8 5 6 8 0 16 2 and 19 2 ml/min × 100 ml tissue (regression significant at $p < 0\ 001$)

In Fig 1 the data from 27 exercise periods are summarized (bottom curve) The dilator response of the resistance vessels in the immediate post contraction period, expressed as post exercise resistance in per cent of the control resistance at rest (100 %), is here plotted against the concomitant increase of venous osmolality The diagram shows classed mean values for ± 1 5 mOsm/kg ranges and the standard deviation The broken line which connects these mean values indicates a similar trend to the abovementioned data from a single subject i e a successively decreasing vascular resistance with increasing osmolality

There was a considerable spread of data in this series of experiments as indicated by the standard deviation This may at least partly be related to a variable ad mixture of blood drained from skin tissue and resting muscle in the samples taken for osmolality determinations Although the observed changes of pO_2, pCO_2, and pH always indicated that the samples contained venous blood mainly from exer cising muscle, some admixture from other regions cannot be excluded Such admix ture was apparently greater in samples taken from a proximal than from a distal venous catheter (see Methods) This was evidenced by the fact that when both these types of catheters were used in the same experiment, simultaneous analyses

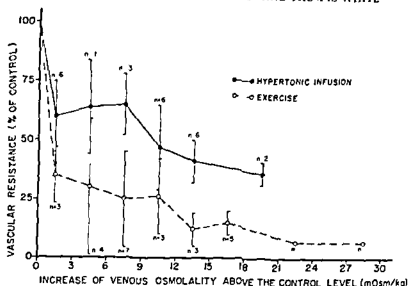


Fig. 1. Vascular resistance in the human forearm expressed in % of control resistance (100 %) in response to exercise (dashed line) and to i.a. hypertonic infusion (solid line) in relation to the concomitant increase above control level of venous osmolality. The plot represents mean values (±1 S.D., vertical lines) for ±1.5 mOsm/kg ranges. n indicates the number of observations in each group.

from both showed more pronounced changes of the blood gases and of osmolality in the samples taken from the distal catheter. For example, in one experiment in *which blood flow during strenuous forearm exercise was 33 ml/min × 100 ml tissue* analyses of samples taken from a proximal vein showed pO_2 26 mm Hg, pCO 88 mm Hg, pH 7.17, and an increase of osmolality by 20 mOsm/kg, whereas samples from a distal catheter showed pO_2 21 mm Hg, pCO_2 94 mm Hg, pH 7.14 and an increase of osmolality by 27 mOsm/kg. In many of the exercise experiments only a proximal catheter was used and the osmolality data from these experiments are included in the diagram of Fig. 1. It is probable, therefore, that the regional osmolar changes during exercise have been underestimated in this diagram.

In an attempt to investigate the possible role of hyperosmolality *per se* for the development of exercise hyperemia, the vascular reactions of the forearm were studied during experimentally increased osmolality. The changes of tissue osmolality in exercise were mimicked by intra-arterial infusion of hypertonic solutions of glucose, xylose or mannitol into the resting forearm. The infusions were adjusted so as to increase venous osmolality to such similar levels as those observed during exercise. Owing to risks of complications (thrombosis etc.), however, the infusion rates and the concentrations of the solutions had to be kept within moderate levels and increases of venous osmolality above 15 mOsm/kg were only *occasionally produced*.

The hypertonic infusions invariably elicited a dilatation of the resistance vessels of the resting forearm, whereas isotonic infusions at the same rates usually caused

no significant changes in regional resistance In the same subject, there was, in general, a progressively decreasing resistance with increasing venous osmolality Most of the observations were obtained with hypertonic glucose infusion, but no major difference with regard to the dilator actions seemed to exist for the other substances used

Fig 1 (upper curve) summarizes 44 observations during hypertonic infusion in 12 subjects In analogy to the exercise experiments, the dilator response of the resistance vessels, expressed in per cent of control resistance, is plotted against the concomitant increase in venous osmolality, and the diagram shows classed mean values and the standard deviation Admittedly, there is a relatively large range of variation but the mean values seem to indicate a relationship between the extent of experimental hyperosmolality and the magnitude of the dilator response

Although the diagram shows quite marked effects of experimental hyperosmolality on vascular resistance in resting human muscle, the evoked decrease of resistance was less pronounced than that elicited in exercise at comparable levels of venous hyperosmolality In the higher range of experimental hyperosmolality, blood flow usually increased to levels about 3 times the flow at rest (in one case about 7 times), whereas strenuous forearm exercise at comparable osmolality and perfusion pressure could lead to a tenfold or occasionally a fifteenfold, increase in flow It is possible, however, that with the experimental approach used in the present study, the vaso dilator effect of hyperosmolality might be underestimated as will be briefly discussed below

In some of the hypertonic infusion experiments a slight vasoconstriction was observed in the contralateral forearm, an effect which might be ascribed to increased sympathetic activity In an attempt to avoid interference of the vasoconstrictor fibres in the test arm during hypertonic infusion one experiment (not included in Fig 1) was performed after ipsilateral blockade of the stellate ganglion The response of the resistance vessels to hypertonic infusion did, however not seem to differ quantitatively from the results presented above

Since there is every reason to believe that the hyperosmolality of exercise at first involves the extravascular space of muscle from where it can influence vascular smooth muscle, an attempt was made to investigate the vasodilator action in resting muscle of experimental tissue hypertonicity when it was not produced *via* the blood stream, but primarily involved the interstitial compartment As a crude approach to this problem the clearance rates of small intramuscular deposits of Xe^{133} were compared when the tracer was dissolved in isotonic and hypertonic medium respectively It is evident that the osmolar level will not be maintained after depositing a small volume of a hypertonic solution in the tissue but will decrease rapidly due to diffusion, and therefore this type of study can at best be considered semi quantitative To be able to create a local hyperosmolality for any length of time the osmolality of the injected test solutions was usually exceeding the level assumed to be reached in exercise (up to about 50 % above isotonicity)

After simultaneous injections of isotonic tracer solution in the anterior tibial

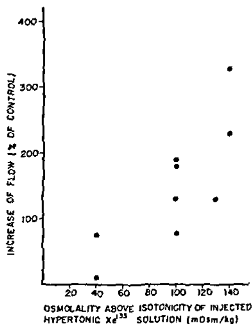


Fig 2 Ordinate Local hyperemia response in human anterior tibial muscle determined with Xe^{133} clearance technique upon i m injection of hypertonic tracer solution The extent to which blood flow observed with hypertonic Xe solution exceeded "isotonic control flow" determined simultaneously with isotonic Xe solution is expressed in % of the "isotonic control flow" Abscissa Osmolality (mOsm/kg) above isotonicity, of the hypertonic Xe solution injected into the muscles

muscle on one side and of hypertonic tracer solution on the other the disappearance curves of Xe^{133} were followed continuously by external monitoring of radioactivity The disappearance curves in both legs were reasonably mono exponential during the first 4 min after injection Blood flow estimated from the clearance constants during this period was always greater during hypertonicity than during isotonicity ("control") The results are summarized in Fig 2 in which the difference between the blood flows after hypertonic and isotonic injections is expressed in per cent of the isotonic control flow Any effect on local blood flow produced by the injection *per se* would seem to be cancelled by expressing the data in this manner It can be seen from the diagram that the local vasodilator response to interstitial hypertonicity was sometimes quite pronounced and that a relation seemed to exist between the degree of hypertonicity of the injected solution and the local hyperemia response

Discussion

The present experiments have shown that skeletal muscle exercise in man leads to an increase of the osmolality in the working muscles which is reflected in the venous effluent This osmolar change is apparently related to the augmented tissue metabolism and to a concomitant release of osmotically active products from the contracting striated muscle fibres (cf Barcroft and Kato 1915) There was a relationship between the magnitude of the evoked exercise hyperemia and the extent to which osmolality increased (cf Fig 1

The hypothesis that the hyperosmolality in the interstitial space that forms the immediate environment of the vascular smooth muscle could lead to smooth muscle relaxation, and hence be a factor in the development of exercise hyperemia was tested after the above basic information was obtained Hyperosmolality was produced experimentally in the resting forearm by intra arterial infusions of hyper tonic solutions at rates which raised the osmolality of the venous effluent to such similar levels as those observed during exercise Such experimental hyperosmolality invariably led to a vasodilatation in the forearm the extent of which seemed related to the degree of hyperosmolality (Fig 1) The fact that a vasodilator response was evoked not only by hypertonic solutions of glucose a normal nutrient but also of non metabolizable substances such as xylose indicated that the effects were related to the change in osmolality *per se* This was supported by the finding that infusion of isotonic glucose solution had little or no effect on the resistance vessels in the forearm despite the fact that this must have created a tissue glucose concentration far above the normal level Intramuscular deposits of Xe^{133} were cleared more rapidly when the tracer was dissolved in a hypertonic than in an isotonic medium This was taken to indicate a hyperemia response also in a situation when the ex perimental hyperosmolality primarily involved the extravascular compartment.

These data suggest that hyperosmolality in working muscle might be a factor that partly explains the exercise vasodilatation in man It is admitted that this con clus on is based mainly on circumstantial evidence but the interpretation seems to be strongly supported by previous observations obtained in animal experiments and in studies of vascular smooth muscle *in vitro* In those investigations it has been possible to test several critical points in greater detail (see Mellander *et al* 1967 Gray *et al* 1968 Johansson and Jonsson 1968) For example *in vivo* experiments showed that the hyperemia observed upon hypertonic infusion was not to any signifi cant extent caused by passive factors such as osmotic dehydration of the vascular walls decreased viscosity of blood due to shrinkage of blood cells etc

Experiments on isolated spontaneously active vascular smooth muscle strongly supported the view that hyperosmolality elicits an active smooth muscle response by exerting a negative chronotropic inotropic and dromotropic action on vascu lar smooth muscle (for details concerning the cellular mechanisms involved see Mellander *et al* 1967 Johansson and Jonsson 1968 Precapillary resistance vessels in skeletal muscle at rest are known to have a high basal myogenic tone and it is possible that the hyperosmolality produced by exercise *in vivo* can lead to inhibi tion of this tone by the mechanisms mentioned and hence to decreased vascular resistance

The present experimental approach does not permit an exact quantitative evalua tion of the role of hyperosmolality in exercise hyperemia in man As mentioned it is likely that in exercise hyperosmolality exerts its action on vascular smooth muscle from the interstitial compartment Therefore it may be questioned whether the degree of the interstitial osmolar change has been correctly estimated during exer cise and further whether extravascular hyperosmolality of similar magnitudes as

in work has been produced in the resting muscle during hypertonic infusion since in both cases the changes of extravascular osmolality were followed only to the extent they were reflected in the venous effluent This is of course a complex question which cannot be answered definitely at present and the discussion will be limited to a few aspects of apparent importance to the problem

If the barrier between the interstitial space and capillary blood prevents the establishment of a transcapillary osmolar equilibrium the extent of extravascular hyperosmolality will be underestimated during exercise but overestimated during hypertonic infusion when determined from samples of the venous effluent The degree to which extravascular osmolality may differ in these two experimental procedures cannot be evaluated in the present study, and the particles responsible for the osmolar changes are of course not the same in the two cases Indirect evidence from animal experiments suggests however that in exercise as well as during hypertonic infusion of the same substances as used here a transcapillary osmotic equilibrium may not be established at least not in short term experiments This was evidenced in terms of marked transcapillary fluid fluxes (osmosis) occurring for considerable length of time after the onset of exercise or hypertonic infusion (*e g* Mellander *et al* 1967)

Another factor that might have led to an underestimation of the role of hyperosmolality in exercise hyperemia in the present experiments was mentioned above (see Results) namely the probable admixture of blood from areas other than exercising muscle in the samples taken for osmolality determination during exercise

Although extravascular osmolality is below but may approach venous osmolality during hypertonic infusions arterial osmolality can at the same time exceed the extravascular osmolality to some extent If the hyperosmolality of arterial blood exerted a direct transintimal influence on the smooth muscles of the arterial vessels the dilator effect of hyperosmolality might have been overestimated in the present experiments Such an effect cannot be entirely ruled out but indirect evidence from animal experiments indicates that during hypertonic infusion the major action of hyperosmolality on vascular smooth muscle takes place from the interstitial space after transcapillary distribution of the solute and that direct transintimal effects on arterial vasculature are small (Mellander *et al* 1967)

Animal experiments performed on the sympathectomized vascular bed in isolated calf muscle in which the parameters can be much more precisely recorded and controlled than in man have suggested a more dominant role of hyperosmolality in exercise hyperemia (*e g* Mellander *et al* 1967 Fig 1) than indicated by the present data on man Fig 1 It is possible that quantitative species differences exist From the foregoing discussion however it appears that hyperosmolality in fact may play a greater role in exercise hyperemia in man than suggested by the present analysis

There is little experimental evidence at present that specific dilator compounds such as acetylcholine ATP bradykinin etc can explain the exercise hyperemia (see Mellander and Johansson 1968) Perhaps hyperosmolality can be looked

upon as a "non specific" dilator principle in the sense that any osmotically active product invading the interstitial space might contribute to the vascular relaxation of exercise The dilator response in exercise might be enhanced by other factors related to augmented metabolism, such as tissue hypoxia and an increased concentration of the potassium ion as mentioned in the introduction but these latter factors have so far not been analysed in any detail in man

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Reflex Responses Induced by Stimulation of Hypoglossal Afferents

By

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Abstract

LINDQUIST, CHR and A MÅRTENSSON *Reflex responses induced by stimulation of hypoglossal afferents* Acta physiol scand 1969 77 234—240

On stimulation of the central end of the cut hypoglossal nerve, multisynaptic reflex discharges were set up in the intrinsic laryngeal muscles and in certain muscles innervated by the facial nerve by contrast, reflex responses were never observed in tongue muscles Activation of the fibers mediating the reflex responses required a stimulus strength 2—4 times that required for activation of the hypoglossal motor fibers On the basis of earlier data on the caliber spectrum of the hypoglossal nerve fibers and on the relation between the threshold values for afferent fibers and motor fibers it is concluded that the hypoglossal afferents belong to the group III fibers The threshold differences also eliminate the possibility of the centrally relayed responses being set up by activation of recurrent collaterals from hypoglossal alpha motor fibers —The possibility that synchronized impulses in the hypoglossal afferents might depolarize central afferent terminals in other nerves to firing level and thus open up reflex connections not operative under physiological conditions is considered and excluded — The types of receptors connected to the high threshold hypoglossal afferents and their functional role are briefly discussed

In previous investigations on dogs (Mårtensson 1963) it was shown that afferent stimulation of the internal laryngeal nerve results in reflex activation of all vocal cord adductors and inhibition of the pre-existent tonic activity in the vocal cord abductor These reflex responses were interpreted as being interoceptive It was also shown that stimulation of individual intact nerves supplying certain intrinsic laryngeal muscles results in a centrally relayed response in the muscle and a closer analysis of this latter response revealed that it represents a recurrent discharge and not a monosynaptic reflex response (Mårtensson 1967) This result is in agreement with morphological investigations which have shown that the dog's intrinsic laryngeal muscles do not contain any muscle spindles (Mårtensson 1964)

The present paper gives an account of experiments on cats showing that centrally relayed responses can be elicited in the intrinsic laryngeal muscles as well as in certain facial muscles on afferent stimulation of the hypoglossal nerve This finding is of interest since contradictory views have been put forward as to the possible presence

of afferent fibers in this nerve Cooper (1954) recorded responses to stretch of the tongue from peripheral hypoglossal branches, but later morphological and physiological investigations have failed to provide conclusive evidence for the presence of organized nerve endings in the tongue muscles (Blom 1960) However, the concept that afferent fibers are present in the hypoglossal nerve may gain some support from earlier experiments by Downman (1939) and Tarkhan and Abou el-Naga (1947) who on stimulation of the hypoglossal nerve in cats and dogs recorded autonomic effects In an investigation on cats, Porter (1965) showed that synaptic potentials generated in hypoglossal motoneurons can be elicited by strong stimuli applied to the hypoglossal nerve, it was suggested that these potentials might be caused by current spread activating lingual nerve afferents or, alternatively, might derive from activation of high threshold afferents in the hypoglossal nerve

The experiments to be described will show that the hypoglossal nerve contains high threshold afferents which are reflexly connected with the nucleus ambiguus as well as with the nucleus of the facial nerve No reflex responses could be evoked in the muscles supplied by the hypoglossal nerve itself

Methods

Tracheotomized cats weighing between 2 5 and 3 5 kg were used for the experiments Anesthesia was induced by ether and maintained by injections of chloralose in doses of 50—60 mg/kg b w , administered through a catheter in the femoral vein

A Grass S 4 stimulator connected to bipolar chlorided silver wire electrodes delivering square wave stimuli of 0 1 msec duration were used for stimulation of the hypoglossal nerve, either intact or cut at the level of the mylohyoid muscle Steel needles insulated to the tip were used for the recording from muscles Recordings were obtained from the following intrinsic laryngeal muscles, i e m cricothyreoideus m cricoarytenoideus posterior m thyreoarytenoideus and m cricoarytenoideus lateralis as well as from perioral muscles platysma and the digastric muscle which are all supplied by the facial nerve and finally from various extrinsic and intrinsic tongue muscles The recording electrodes were connected to a Tektronix 502 dual beam oscilloscope and a loudspeaker via a cathode follower and a Grass P 6 amplifier

Results

In the main series of experiments the proximal part of the cut hypoglossal nerve was stimulated and recordings made from various intrinsic laryngeal muscles and from certain muscles supplied by the facial nerve

Fig 1 presents typical recordings from such experiments showing how a sufficiently strong stimulus (cf below) gives rise to discharges in the cricoarytenoideus posterior cricothyreoideus and a perioral muscle Similar reflex discharges could also be obtained in other laryngeal and facial muscles tested (Fig 2 and 3) The

Fig 1 Reflex discharges in response to stimulation of the central end of the cut hypoglossal nerve recorded in *A* m cricoarytenoideus posterior *B* m cricothyreoideus and *C* m orbicularis oris Time bar 10 msec

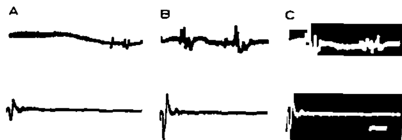


Fig 2 Reflex discharges in the thyroarytenoid muscle (upper beam) and direct action potentials in the styloglossus (lower beam) in response to stimulation of the intact hypoglossal nerve Full description in text Time bar 10 msec

latency between stimulation and reflex discharge in the muscles tested was generally around 10—15 msec Estimates of the central relay times give values corresponding to those for multisynaptic reflexes

A possible activation of nearby afferent nerves by direct spread of stimulus current could be ruled out since no reflexes were obtained after crushing the hypoglossal nerve central to the stimulating electrodes

A special series of experiments was performed to find out if stimulation of the hypoglossal nerve elicited any reflex responses in the muscles supplied by this nerve Fig 2 illustrates an experiment in which the intact hypoglossal nerve was stimulated at different current strengths The lower beam shows the direct response obtained in a tongue muscle and the upper beam for comparison the simultaneous reflex discharges in the thyroarytenoid No reflex discharges can be observed in the tongue muscle whether the stimulus current is submaximal (*A*) maximal (*B*) or supramaximal (*C*) for eliciting the direct action potential When the whole intact nerve is stimulated as in this type of experiment it has to be taken into account that an antidromic volley may cause a postexcitatory depression in the hypoglossal motor nucleus However the same negative result was obtained in experiments using stimulation of a cut hypoglossal nerve branch and recording from tongue muscles innervated by intact branches

Simultaneously with the maximal direct responses in Fig 2 *B* and *C* a reflex discharge is set up in the thyroarytenoid muscle of the same latency as the responses obtained on stimulation of the central end of the cut hypoglossal nerve (*cf* Fig 1) In addition to this reflex a discharge of longer latency is elicited which appears even *with the submaximal direct response in the tongue muscle (Fig 2 A)* The size of the long latency discharge increases until a maximal direct response is obtained in the tongue muscle (*B*) but on further increase in stimulus strength there is no further rise in amplitude of this response (*C*) The long latency of this reflex discharge and fact that its amplitude increased fairly parallel with that of the direct response indicates that it was caused by a twitch contraction in the tongue giving rise to an

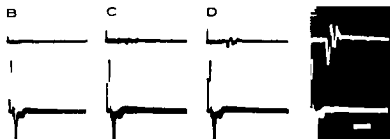


Fig 3 Reflex discharges in the digastric muscle (upper beam) and direct action potentials in the styloglossus (lower beam) in response to stimulation of the intact hypoglossal nerve Full description in text Time bar 10 msec

afferent volley in the lingual afferents (*cf* Blom 1960, Porter 1966) This was borne out b experiments in which the secondary response could no longer be obtained after curarization

In order to get an idea of the type of afferent fibers mediating the primary reflex response the stimulus threshold for a response in the hypoglossal afferents using the reflex discharge as index was compared to the threshold for a response in the hypoglossal alpha motor fibers as mirrored by the directly evoked tongue muscle action potential The recordings in Fig 3 may serve as an illustration of this type of experiment The lower beam represents the direct action potential evoked in the styloglossus in response to stimulation of the intact hypoglossal nerve and the upper beam the reflex discharge recorded in the digastric muscle As the stimulus strength is increased the thresholds for a submaximal (*A*) and a maximal (*B*) direct response are gradually attained but not until the strength is twice that required for a maximal direct action potential has the threshold for a reflex discharge in the digastric been reached (*C*) As the stimulus is further increased to about 3 times (*D*) and 6 times (*E*) that in *B* there is a rise in amplitude of the reflex discharges Similar differences were observed also in other muscles when comparing the stimuli required to elicit direct and reflex responses The stimulus strength necessary for a reflex response corresponded to or exceeded that yielding a maximal direct action potential and there was invariably an appreciable rise in amplitude of the reflex discharge on further increase of the stimulus strength On an average the threshold values for eliciting a reflex discharge were 2—4 times higher than those for alpha motor fibers The reflex responses are thus transmitted through hypoglossal afferents of comparatively high thresholds

In a minor series of experiments the effects of conditioning volleys in the hypoglossal nerve on reflexes elicited by stimulation of different sensory nerves were tested Multisynaptic reflexes evoked by stimulation of the internal laryngeal nerve could then be depressed for up to 200—300 msec even when the strength of the conditioning stimuli was below threshold for hypoglossal reflexes Reflexes in

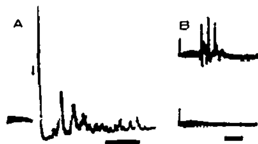


Fig 4 *A*, typical dorsal root reflex recorded in the lingual nerve in response to stimulation applied to the same nerve *B* reflex discharge in the thyroarytenoid muscle (upper beam) in response to stimulation applied to *the central part of the cut hypoglossal* nerve No dorsal root reflex is elicited in the lingual nerve (lower beam Time bars 10 msec

laryngeal muscles as well as in the digastric muscle set up by lingual nerve stimulation were similarly depressed The inhibitory effect typically diminished rapidly on increasing stimulus frequency

Further research is necessary before the possible mechanism behind these inhibitory phenomena can be identified but several of its features are characteristic of a presynaptic inhibitory mechanism (*cf* Eccles 1964) Presynaptic inhibition has recently been observed in the trigeminal and vagal nuclei (Darian Smith 1965 Rudomin 1967) A mechanism of this type would imply that the hypoglossal afferents should be linked to interneurons terminating on primary afferents in the lingual and internal laryngeal nerves

In this context we would like to draw attention to this type of connections as a possible pathway also for transmission of artefactual reflexes under the less physiological conditions represented by experiments using artificial stimulation such as the present ones Whereas a more disperse afferent volley would only give the local depolarization of the central afferent terminal required for operation of the inhibitory mechanism the synchronous activation of the hypoglossal afferents in these reflex studies might set up an abnormal series of impulses in the interneuron pool which when converging on the presynaptic terminals could cause summed depolarizations sufficient to initiate impulses In such a situation a trigeminal dorsal root reflex should be generated (*cf* Brooks and Koizumi 1956) but obviously the impulses can also travel in the centripetal direction and open up reflex paths which are not operative under normal conditions To ascertain that the reflexes described in this paper do not derive from a mechanism of this type a few experiments were performed in which simultaneously with the recording of the reflex a recording was also made from the lingual nerve or the internal laryngeal nerve in order to see whether or not a dorsal root reflex appeared Fig 4 *A* serves to illustrate the characteristic appearance of a dorsal root reflex in the lingual nerve set up by afferent stimulation of the same nerve In Fig 4 *B* (lower beam) no dorsal root reflex is elicited in the lingual nerve although the typical high threshold reflex response is recorded in the thyroarytenoid muscle Hence the high threshold hypoglossal reflexes not result from non physiological activation of the lingual or internal laryngeal ve of the type outlined above

Discussion

Some possible sources of error had to be excluded before it could be established that the activation of the various laryngeal and facial muscles is caused by stimulation of high threshold hypoglossal afferents setting up multisynaptic reflexes A possible activation of other afferents by current spread—or by unintentional stimulation of tongue muscles giving rise to muscle twitches and subsequent activation of lingual mechanoreceptors—could be ruled out by the control experiments described Another possibility to be considered was that antidromic stimulation of motor fibers in the hypoglossal nerve might have activated recurrent collaterals from hypoglossal motoneurons with connections to other nuclei However experiments of the type illustrated in Fig 3 showed that the threshold for the centrally relayed responses was higher than that for activation of hypoglossal motor fibers and this eliminates the possibility that the relayed responses might be set up by activation of recurrent collaterals (*cf* Porter 1965) The question was finally raised whether synchronous impulses in the hypoglossal afferents might depolarize central afferent terminals in other nerves to firing level and thus open up artifical reflex connections but experiments designed to test this possibility did not provide any support for such an explanation

It remains to discuss what types of receptors may mediate activity via the high threshold afferents and what functional role they may play Thresholds for the hypoglossal afferents were estimated to be 2—4 times higher than those for hypoglossal motor fibers Blom (1960) has shown that the hypoglossal nerve has a caliber spectrum differing from that of skeletal muscle nerves in that it has a single sharp peak around 6—8 μ and very few fibers above 10 μ In view of the relative thresholds of motor fibers and afferent fibers the afferents should thus belong in the group III range (1—6 μ) Afferent group III fibers from skeletal muscle are considered to be connected to pressure and pain receptors (Paintal 1960) Owing to the specific diameter spectrum of the hypoglossal nerve however the relationship between fiber group and function may not be the same for this nerve

Porter (1966) studied afferent discharges from superficial and presumedly deeply situated mechanoreceptors in the cat s tongue In his experiments about half of the receptors responded to muscle twitches However activity from these mechanoreceptors could only be recorded from the lingual nerve no mechanoreceptor fibers could be identified in the hypoglossal nerve This is in keeping with our findings that the hypoglossal afferents could not be activated by tongue muscle twitches as is evident from the experiment using stimulation of the intact hypoglossal nerve illustrated in Fig 2 which shows that the high threshold hypoglossal reflexes do not appear until the stimulus strength exceeds that required for eliciting a maximal twitch in the tongue muscles

In a short communication which became known to us during the preparation of this paper Sauerland and Mizuno (1968 showed that multisynaptic reflex effects can be recorded in the recurrent laryngeal nerve on stimulation of the central end of the cut hypoglossal nerve They also showed that these reflexes could no longer

be elicited after section of the vagal rootlets and drew the conclusion that hypoglossal afferents in their central course join the vagus nerve In another recent study (Nakamura 1968) it has however been shown that effects of afferent hypoglossal nerve stimulation on masseteric monosynaptic reflexes persisted even after severance of the vagal-accessory as well as the cervical nerve roots but were abolished after *section of the hypoglossal nerve root The location of the cell bodies of the hypo*glossal afferents is *still* an open question which however should have no direct bearing on the interpretation of the results presented in this paper

As far as the functional role of the hypoglossal afferents is concerned it seems unlikely that they should be involved in the proprioceptive control of the tongue muscles since their receptors do not respond to tongue muscle twitches and since on stimulation they do not set up reflexes in the tongue muscles themselves They may *instead serve to coordinate activity in some of the muscles innervated by the nucleus* ambiguus and the facial motor nucleus Further research is obviously required to clarify this point as well as the question of what types of receptors are involved

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The Effect of Acetazolamide upon Tissue Concentrations of Bicarbonate, Lactate, and Pyruvate in the Rat Brain

By

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Abstract

Kjällquist, A, M Nardini and B K Siesjö *The effect of acetazolamide upon tissue concentrations of bicarbonate, lactate, and pyruvate in the rat brain* Acta physiol scand 1969 77 241—251

The effect of various doses of acetazolamide upon acid base parameters in brain tissue, cisternal CSF and arterial plasma was studied in rats, anesthetized with phenobarbital or nitrous oxide The results confirmed older findings that carbonic anhydrase inhibition increases the CSF/plasma ratio for bicarbonate, and decreases the corresponding chloride ratio, and showed that the CSF pH remains unchanged during at least 6 hrs in spite of a marked plasma acidosis The tissue bicarbonate was found to be significantly, but transiently, increased, and since the CSF bicarbonate was essentially unchanged, the increase in bicarbonate must have occurred in the intracellular phase, i e an alkaline shift in the cells However, both the lactate and the pyruvate concentrations in the tissue were decreased after acetazolamide, suggesting an acidosis It is discussed if these controversial findings indicate that carbonic anhydrase inhibition gives rise to a carbonic acid acidosis in the brain

The effects of carbonic anhydrase inhibition on secretory functions and on ion transport in several different organs are well known (see review by Maren 1967), and as the occurrence of carbonic anhydrase in glial cells in the brain has been established (Giacobini 1962), it has been assumed that carbonic anhydrase-catalyzed reactions are involved in hydrogen ion transport in the brain However since a study of the acid base metabolism of a tissue requires that the tissue CO_2 tension is known, and since carbonic anhydrase inhibitors like acetazolamide lead to a disequilibrium in the CO_2 buffer systems of the organism precluding accurate assessment of blood CO_2 tensions, the effect of carbonic anhydrase inhibition on tissue acid-base parameters is poorly understood

Recently we reported measurements of blood and brain tissue CO_2 tensions in rats and cats given various doses of acetazolamide (Brzezinski Kjällquist and Siesjö 1967) The tissue CO_2 tension were either measured directly with a surface CO_2

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electrode, or were derived from the CO_2 tension in cisternal CSF The relations obtained in the work quoted make it possible to study the effects of acetazolamide on tissue acid-base parameters in the brain In a preliminary paper we reported that carbonic anhydrase inhibition in the rat leads to an increase in the tissue bicarbonate concentration in the brain, which seemed to be unrelated to any increase in the tissue CO_2 tension (Kjällquist and Siesjö 1966).

The present paper gives details of the measurements of the tissue and CSF bicarbonate concentration in rats given 50—200 mg acetazolamide per kg, and studied at various times after injections of the drug (1—10 hrs) In some of the groups chloride was measured in plasma and in cisternal CSF, and in some of the later experiments the tissue concentrations of lactate and pyruvate were measured Both the lactate and pyruvate concentrations of tissues are known to decrease in acidosis and to increase in alkalosis (Scheuer and Berry 1967, Leusen, Weyne and Demeester 1968, Granholm and Siesjö 1968, Kjällquist, Nardini and Siesjö 1969), and lactate/pyruvate measurements can thus tentatively be used to assess the direction of a pH change in tissues Most of the earlier experiments in the present material were made on rats anesthetized with phenobarbital, but since the distribution of this drug can be altered by acid-base changes (Waddell and Butler 1957, Goldberg Barlow and Roth 1961), later control measurements were made under nitrous oxide anesthesia The latter was found to give a much smaller variability in the tissue concentrations of lactate and pyruvate and was therefore used in all experiments in which these metabolites were measured

Methods

The experiments were carried out on male Wistar rats weighing 250—450 g Most of the rats were anesthetized with i p phenobarbital (150 mg/kg b w) tracheotomized and allowed to breathe air or CO_2 containing gas mixtures spontaneously In the nitrous oxide group of animals anesthesia was induced with divinyl ether and continued with 70 % nitrous oxide and 30 % oxygen after a quick tracheotomy These animals were immobilized with tubocurarine chloride and ventilated with a Palmer miniature respirator By hyperventilation or by addition of CO_2 different levels of arterial P_{CO_2} (20 [illegible]90 mm Hg) could be obtained In all animals the blood pressure was measured via a cannula in one femoral artery using a capacitive electromanometer, and the rectal temperature was measured with an electrothermometer

The operative procedures and the methods used to sample and analyse blood cisternal CSF and brain tissue have been described in previous communications from the laboratory (for procedures and further references see Siesjö and Pontén 1966 Pontén 1966 Kjällquist Nardini and Siesjö 1969) In general pattern the analytical procedures involved that arterial blood was sampled anaerobically and that measurements were made of the pH the P_{CO_2} and the hemoglobin concentration while the plasma chloride concentration was measured after anaerobic centrifugation Cerebrospinal fluid (CSF) was withdrawn from the cisterna magna and was either analysed for the total CO_2 content or for the chloride concentration Brain tissue was frozen in situ and analysed for the total CO_2 content and the water content and for the lactate and pyruvate concentration using extraction of the tissue at −15° C (Granholm Kaasik Nilsson and Siesjö 1968

The mean tissue (and the CSF) CO_2 tension was calculated from the measured arterial CO_2 tension using the relations described in a previous communication from the laboratory (P [illegible] frase inhib [illegible] all just at [illegible] with nitrous [illegible] (Kjällquist

[illegible] have shown that these relations are valid also in hyper [illegible]
[illegible] Demeester and Siesjö 1969)

In all experiments the mean tissue bicarbonate concentration was obtained from the measured total CO content by subtracting the amount of bicarbonate contained in the blood volume assumed to be 3 per cent of the tissue weight, and the amount of physically dissolved CO_2 calculated as the product $P_{tCO_2} \times 0.0297$ mmoles/kg of wet tissue In order to be able to compare differences in the tissue bicarbonate concentration which were not due to differences in the CO tension the standard bicarbonate concentration ($P_{tCO_2}=40$ mm Hg) was calculated However the changes in the tissue buffer base concentration were also calculated and expressed as a base excess value according to

$$BE = B\,B - 35\,7$$

where 35 7 is the normal buffer base (Ponten 1966) and BB is the actual buffer base in the expe imental situation calculated as

$$BB = \frac{(HCO_3)\ 35}{(HCO_3) + P_tCO_2\ S} + (HCO_3)$$

(Ponten 1966) However, since Ponten s buffer lin ... with various CO_2 tension for 30 min and since ... exposed to a slightly increased CO tension for a ... of phenobarbital anesthetized animals were analyse ... 3 hrs while unanesthetized. The acetazolamide treated animals were thus also compared to a buffer line derived from normocapnic and from these hypercapnic animals (see Fig 1)

The carbonic anhydrase inhibitor acetazolamide (Diamox, American Cyanamide Co New York) was given as a 10 % solution of the sodium salt in distilled water The drug was given i p either in a single dose of 50 mg/kg or in repeated doses of the same size There were two main groups of animals In the first main group a single dose was given, and the animals were studied 1 3 or 6 hrs after the injection (group C D and E in Table II and III) In the second main group a high acetazolamide concentration was upheld by means of repeated injections during 3 6 and 10 hrs respectively (group F G and H) In these groups injections were given at 0 and 2 hrs at 0 2 and 4 hrs and at 0 3 6 and 9 hrs respectively

Since the operative procedures and the attainment of a steady state after the operation took about 30—35 minutes the animals were anesthetized 45 min before the end of the intended exposure time

Control experiments were obtained by using uninjected rats (group A) or by injecting an equimolar dose of 2 acetamino-1 3 4 thiadiazole 5(N t butyl)-sulphonamide (CL 13850 American Cyanamide Co New York) This drug which is chemically similar to acetazolamide but which does not inhibit carbonic anhydrase has been used as a control substance for acetazolamide (Wistrand Nechay and Maren 1961 *cf* Brzezinski *et al* 1967) The control substance was administered in two doses during 3 hrs or in three doses during 6 hrs (Group B)

In order to obtain a rough quantitative measure of the acetazolamide concentration in the brain tissue the micromethod of Maren (1960) was used to assess the concentration of the drug in blood and in homogenized brain tissue Before the removal of the supratentorial parts of the rat brains the tissues were perfused with isotonic Krebs Henseleit solution from the aorta The perfusion was continued until the fluid emerging from the cut jugular veins was visually hemoglobin free The tissue was then homogenized with distilled water and placed in a water bath at 100° C for 5 min in order to inactivate the carbonic anhydrase Maren *et al* 1954) The degree of inhibition was calculated as the fractional inhibition $i = I/(I + K_I)$ where I is the total concentration of acetazolamide found by analysis and K_I is the dissociation constant of the enzyme inhibitor complex 6 4 10 according to Maren 1962 a)

In part of the present study only blood and tissue parameters were measured while in some groups (B C E F and G) 40—60 µl of CSF were withdrawn prior to the freezing of the tissue *in situ* It was found by statistical analysis that there were no significant differences between tissue values obtained with and without collection of CSF In order to avoid withdrawal of CSF in amounts larger than 50—70 µl the total CO_2 content the chloride concentration or the lactate and pyruvate concentrations of the CSF were measured on separate animals However in the majority of the experiments the tissue was frozen and the total tissue CO_2 content was measured The numbers of experiments relating to the measurements of ... these experiments where the total CO_2 content ... bonate concentration was calculated by sub... an extracellular volume which was assumed ... Nardini and Siesjö 1969 Using this value for the bicarbonate concentration in the intracellular water phase an equivalent intracellular pH was calculated under the assumption that the apparent pK value of 6 12 is valid for the actual carbon dioxide tension in the tissue

TABLE I Rats, phenobarbital anaesthesia Concentrations of acetazolamide in whole blood and in homogenized whole brain tissue Calculations of fractional carbonic anhydrase inhibition is described in the text P values represent level of significance for differences of the respective group from the 50 mg/kg group

Dose (mg/kg) and exposure time	Whole blood		Brain		
	μg/ml	μM/1	μg/g	μM/kg	Fractional inhibition
50 mg/kg 1 hr	48 ±7 (4)	215 ± 32	7 8 ± 0 9 (4)	35 5 ± 3 9	0 9981 ± 0 0002
100 mg/kg 3 hrs	32 ± 7 (4)	142 ± 31	10 2 ± 2 2 (3)	46 1 ± 10 1	0 9981 ± 0 0001 P < 0 0)
150 mg/kg 6 hrs	40 ± 4 (5)	180 ± 17	14 3 ± 1 3 (5) P < 0 01	64 4 ± 6 0	0 9990 ± 0 0001 P < 0 01

TABLE II Influence of acetazolamide on acid/base parameters and chloride concentration in ... mals within brackets

Dose (mg/kg) and exposure time		Arterial plasma			
		pH	$PaCO_2$ mmHg	HCO_3^- mEq/l	Stand HCO_3^- mEq/l
A	Uninjected	7 42 (13) 0 01	40 6 (13) ± 0 9	25 3 (13) ± 0 7	2[illegible] (13) ± 0 6
B	CL 13 850	7 41 (12) ± 0 01	41 5 (12) ± 0 8	25 2 (12) ± 0 7	2[illegible] 0 (12) 0 6
C	Acetazolamide 50 mg kg 1 hr	7 35 (5) 0 02	45 8 (5) 2 4	23 4 (5) ± 1 1	22 3 (5) 1 0
D	3 hrs	7 29 (6 0 01	41 7 (6) 1 1	19 3 (6) ± 0 6	18 9 (6) 0 5
E	6 hrs	7 32 (12 0 01	37 8 (12) 0 9	18 7 (12) 0 6	19 1 (12) 0 6
F	100 mg kg 3 hrs	7 27 28 0 01	43 9 (28 0 7	19 2 (28) 0 3	18 4 (28) ± 0 2
G	150 mg kg 6 hrs	7 2 10 0 02	42 0 (10 1 8	17 4 (10) ± 0 7	17 1 (10) ± 0 7
	200 mg kg 10 hrs	7 23 0 02	41 [illegible] (6 2 0	16 7 (7) 0 5	16 3 (7) ± 0 5

Results

The acetazolamide concentrations measured in whole blood and in brain tissues as well as the measured and derived acid base parameters of arterial blood, brain tissue and CSF from the control animals as well as from animals given various doses of acetazolamide, are given in Table I—IV The effect of acetazolamide upon the extra- and intracellular bicarbonate concentrations is exemplified in Fig 1, while the effect on the tissue lactate and pyruvate concentrations is shown in Fig 2

Acetazolamide concentrations Table I shows the acetazolamide concentrations measured in whole blood and in the supratentorial parts of the brain The table shows that there was an appreciable acetazolamide concentration in the brain already 1 hr after a single dose of 50 mg/kg and that the concentration increased in the groups given 100 and 150 mg/kg respectively There are reasons to believe that the tissue acetazolamide concentrations in the groups studied (and in the 10 hrs group) are sufficient to cause a functional inhibition of the tissue carbonic anhydrase (see Maren 1967) Since no correction was applied for the outwashing of free

arterial blood and cisternal CSF (means ± s e) Rats, phenobarbital anaesthesia Number of ani

	CSF				
Cl meq/l	TCO_2 mM/kg	PCO_2 mmHg	HCO_3 mEq/kg	pH	Cl mEq/kg
10? (2)	28 8 (2)	—	—	—	130 (2)
105 1 (6) ± 1 1	28 3 (6) ± 0 5	47 8 (6) ± 1 4	26 8 (6) ± 0 4	7 38 (6) 0 01	129 8 (6) ± 2 1
—	30 1 (4) ± 0 9	50 3 (4) ± 2 3	28 5 (4) ± 0 9	7 38 (4) 0 03	—
—	—	—	—	—	—
[illegible] ± 1 7	[illegible] ± 0 4	[illegible] ± 1 7	[illegible] ± 0 4	[illegible] ± 0 01	[illegible] 2 0
110 9 (10) ± 0 7	28 6 (7) ± 0 5	49 5 (7 ± 1 5	27 1 (7 ± 0 5	7 37 0 01	124 ? 10) ± 0 9
—	26 8 5 ± 0 3	44 7 (5) ± 1 1	25 4 (5) ± 0 3	39 5) 0 01	—
—	26 2 (6) ± 0 7	46 9 (6) ± 0 8	24 7 (6) ± 0 6	35 6) 0 01	—

TABLE I Rats, phenobarbital anaesthesia Concentrations of acetazolamide in whole blood and in homogenized whole brain tissue Calculations of fractional carbonic anhydrase inhibition is described in the text P values represent level of significance for differences of the respective group from the 50 mg/kg group

Dose (mg/kg) and exposure time	Whole blood		Brain		
	μg/ml	μM/l	μg/g	μM/kg	Fractional inhibition
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150 mg/kg 6 hrs	40±4 (5)	180±17	14 3±1 3 (5) P<0 01	64 4±6 0	0 9990±0 0001 P<0 01

TABLE II Influence of acetazolamide on acid/base parameters and chloride concentrations in mals within brackets

	Dose (mg/kg) and exposure time	Arterial plasma			
		pH	$PaCO_2$ mmHg	HCO_3 mEq/l	Stand HCO_3^- mEq/l
A	Uninjected	7 42 (13) ± 0 01	40 6 (13) ± 0 9	25 3 (13) ± 0 7	25 5 (13) ± 0 6
B	CL 13 850	7 41 (12) ± 0 01	41 5 (12) ± 0 8	25 2 (12) ± 0 7	25 0 (12) ± 0 6
C	Acetazolamide 50 mg/kg 1 hr	7 35 (5) ± 0 02	45 8 (5) ± 2 4	23 4 (5) ± 1 4	22 3 (5) ± 1 0
D	3 hrs	7 29 (6) ± 0 01	41 7 (6) ± 1 1	19 3 (6) ± 0 6	18 9 (6) ± 0 5
E	6 hrs	7 32 (12) ± 0 01	37 8 (12) ± 0 9	18 7 (12) ± 0 6	19 1 (12) ± 0 6
F	100 mg/kg 3 hrs	7 27 (28) ± 0 01	43 9 (28) ± 0 7	19 2 (28) ± 0 3	18 4 (28) ± 0 2
G	150 mg/kg 6 hrs	7 23 (10) ± 0 02	42 0 (10) ± 1 8	17 4 (10) ± 0 7	17 1 (10) ± 0 7
	200 mg/kg 10 hrs	7 23 (7) ± 0 02	41 6 (6) ± 2 0	16 7 (7) ± 0 5	16 3 (7) ± 0 5

TABLE IV Acid/base changes in rat brain tissue after acetazolamide The same groups as in table II Maximal increase in buffer base and bicarbonate concentration appeared after 3 hours inhibition of carbonic anhydrase (group F) but after 10 hrs there were no changes from the control values (group A or B)

Dose (mg kg) and exposure time		Brain tissue					
		T CO_2 mM/kg	P CO_2 mmHg	HCO_3 mEq/kg	HCO_3 mEq/kg H_2O	Stand HCO_3 mEq/kg H_2O	Base excess mEq kg
A	Uninjected	14.6 (10) ± 0.2	46.3 ± 1.0	13.0 ± 0.2	16.6 ± 0.2	15.8 ± 0.1	−0.4 ± 0.3
B	CL 13850	14.7 (5) ± 0.2	47.3 ± 0.8	13.2 ± 0.2	16.7 ± 0.3	15.8 ± 0.2	−0.4 ± 0.5
C	Acetazolamide 50 mg/kg 1 hr	15.8 (4) ± 0.4	52.2 ± 2.9	14.2 ± 0.5	18.1 ± 0.4	16.8 ± 0.2	+0.9 ± 0.3
D	3 hrs	15.6 (6) ± 0.2	47.9 ± 1.1	14.1 ± 0.2	18.0 ± 0.3	17.0 ± 0.2	+1.4 ± 0.3
E	6 hrs	14.6 (9) ± 0.4	45.0 ± 0.9	13.1 ± 0.3	16.7 ± 0.4	16.0 ± 0.3	−0.1 ± 0.5
F	100 mg/kg 3 hrs	16.1 (19) ± 0.2	49.9 ± 0.7	14.6 ± 0.1	18.6 ± 0.2	17.4 ± 0.2	+2.0 ± 0.2
G	150 mg/kg 6 hrs	15.8 (7) ± 0.2	50.2 ± 1.9	14.3 ± 0.2	18.2 ± 0.4	16.9 ± 0.4	+1.2 ± 0.5
H	200 mg/kg 10 hrs	14.5 (7) ± 0.2	47.7 ± 1.7	13.1 ± 0.3	16.8 ± 0.3	15.9 ± 0.5	−0.2 ± 0.6

1960) in showing an unchanged or only moderately lowered CSF bicarbonate concentration in spite of a generally highly significant decrease in the plasma bicarbonate (Table II), and in showing progressive decreases and increases of the CSF/plasma chloride and bicarbonate ratios respectively (Table III) It should be remarked that in spite of the decreased plasma pH and bicarbonate concentration the calculated CSF pH was significantly altered only in the 10 hrs group ($0.01 < p < 0.001$)

Brain tissues The dominant changes in the tissue parameters after acetazolamide were increases in the total CO content and in the calculated buffer base concentration as well as decreases in the lactate and pyruvate concentrations Table IV shows that there was a significant increase in the buffer base concentration 3 hrs after a single dose of acetazolamide of 50 mg/kg but no significant change 6 hrs after such a dose After 100 mg/kg there was a highly significant increase in the

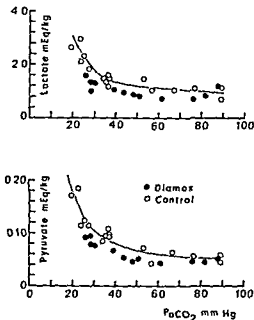


Fig 2 The relation between the arterial CO_2 tension and the lactate and pyruvate concentrations of brain tissue in uninjected rats (unfilled circles) and in rats injected with acetazolamide in a dose of 100 mg/kg

buffer base concentration but this increase appeared to vanish in spite of repeated administrations of acetazolamide and it had completely disappeared in group H

Fig 1 shows the tissue bicarbonate concentrations after acetazolamide as compared to the 3 hr buffer line (see Methods) The increase was even more apparent when the calculated intracellular bicarbonate concentrations were compared During acetazolamide the calculated equivalent intracellular pH was increased in comparison to the control animals (CL 13 850) Thus the control group had a mean value of 7 13 + 0 01 while the pH in the groups F G and H was 7 16 ± 0 01 7 18 ± 0 01 and 7 15 + 0 02 respectively The increase was significant in group F ($p < 0.05$) and group G ($p < 0.01$)

The tissue lactate and pyruvate concentrations were measured in nitrous oxide animals given two doses of acetazolamide of 50 mg/kg each (cf group F after that control measurements had shown that the same increase in the tissue bicarbonate concentration was obtained as in the phenobarbital series (Kjallquist Messeter and Siesjo 1969 Fig 2 shows that acetazolamide led to significant decreases in both the lactate and pyruvate concentrations as compared to uninjected nitrous oxide rats

Discussion

There is evidence that the enzyme carbonic anhydrase which catalyzes the hydration of carbon dioxide and the dehydration of carbonic acid is involved in active transport of ions In e g the kidney and in the pancreas secretory exchanges of H and HCO ions are affected by the administration of carbonic anhydrase inhibitors (review by Maren 1967 In the brain the enzyme is localized to plexus

chorioideus and to glial cells (Giacobini 1962), and it is only logical that it has been assumed to be involved in transport of ions at these sites. It has been shown that carbonic anhydrase inhibitors like acetazolamide (Diamox) reduce the rate of CSF production and the rate of turnover of sodium ions between the brain and blood (Oppelt *et al.* 1964, Davson and Luck 1957, Koch and Woodbury 1960). There is also some evidence that ion exchanges involved in excitability changes are affected by carbonic anhydrase inhibitors since these drugs increase the threshold to experimental convulsions (Woodbury and Karler 1960) and have been successfully used in the treatment of epileptic seizures (for references see Maren 1967). However the only direct indication of an involvment of carbonic anhydrase in H and HCO_3^- exchanges in the brain is an increase in the CSF/plasma ratio for HCO_3 after acetazolamide (Davson and Luck 1957, Maren and Robinson 1960, Maren 1962 b, Kronenberg and Cain 1968) but these findings cannot be taken to indicate an interference with active transport as long as information is lacking on electrochemical potential differences between the compartments. On the whole there is no clear evidence that acetazolamide affects the steady state distribution of these ions in a way suggestive of an interference with active transport and measurements of the bicarbonate concentration of rat brain tissue relatively long periods after moderate doses of acetazolamide did not reveal any significant changes (Koch and Woodbury 1960).

The present results have shown that the administration of relatively high doses of acetazolamide acutely leads to a significant increase in the tissue bicarbonate concentration and to significant decreases in the tissue lactate and pyruvate concentrations. The measured concentrations of acetazolamide in the tissue and the calculated degrees of fractional inhibition indicated that about 99.9 % of the enzyme was inhibited as calculated for the whole brain. Presumably this is valid also for the glial and plexus cells and in analogy with findings in other organs this degree of inhibition should be sufficient to give rise to functional changes (Maren 1963). An analysis of the increase in the bicarbonate concentration with the help of the *in vivo* buffer curve of brain tissue shows that it represents an apparent increase in the buffer base, i.e. an increase in the bicarbonate which is unrelated to the measured or calculated tissue CO_2 tension. The maximal increases in the base excess value was 1—2 mEq/kg of tissue water. However if allowance is made for the fact that the CSF bicarbonate concentration was unchanged and if it is assumed that the CSF bicarbonate concentration represents a homogeneous bicarbonate concentration in a 12 % extra-cellular fluid the increase in the intracellular base excess can be assessed to 2—3 mEq/kg of intracellular water. The increase in the intracellular bicarbonate concentration was apparent already one hour after a single acetazolamide dose of 50 mg per kg but was no longer present six hours after such a dose. The results obtained after six and ten hours respectively of repeated injections of azetazolamide indicated that the eff ct n th ti sue bicarbonate concentration was a transient one. The tissue lactate and pyruvate concentrations were measured at only one exposure time but the re ult urequiv cally showed that the tis nd

pyruvate concentrations were decreased at a time when there was a highly significant increase in the tissue bicarbonate concentration (group F)

As this increase in the bicarbonate concentration should represent a real increase in buffer base, the 'intracellular pH' should have shifted in the alkaline direction However, if the observed decrease in the tissue lactate and pyruvate concentrations are due to an intracellular acidosis which normally seems to be the case (Leusen Weyne and Demeester 1968 see also Granholm and Siesjo 1968, Kjallquist Nardini and Siesjo 1969) the present increase in the tissue bicarbonate concentration should be associated with a decrease in the 'intracellular pH'

An attracting way of explaining the present results is the suggestion put forward by Severinghaus and Cotev (1968) that acetazolamide leads to an intracellular H_2CO_3 acidosis Their hypothesis requires that H_2CO_3 and not CO_2 is formed in the decarboxylation reactions in the brain A H_2CO_3 acidosis of that kind can evidently explain both the increased bicarbonate concentration and the decreased lactate and pyruvate concentrations in the present experiments Under such circumstances there would be a disequilibrium between the H_2CO_3 concentration and the CO_2 tension measured In other words the tissue CO_2 tension would be underestimated in that the equilibrium CO_2 tension corresponding to the actual H_2CO_3 concentration should be 10 to 15 mm higher This figure can be obtained both from the measured bicarbonate concentration assuming a constant buffer base value and from the lactate pyruvate curves of Fig 2 If this is the actual mechanism under carbonic anhydrase inhibition the bicarbonate concentration calculated for group F should be associated with a decrease in 'intracellular pH' and not with an increase in pH

Attractive as it may be the hypothesis of Severinghaus and Cotev (1968 cannot be accepted without doubts There are thus strong experimental indications that CO_2 and not H_2CO_3 is the normal end product of decarboxylations in the brain (Natour and Palmer 1961) A continuous H_2CO_3 acidosis is also difficult to reconcile with the present observation that the tissue bicarbonate concentration returns to normal after some hours of continuous carbonic anhydrase inhibition provided this finding does not imply that the intracellular buffer base slowly decreases under such circumstances If this is the case carbonic anhydrase must be ascribed a definite role for the regulation of the intracellular hydrogen ion activities Thus further work on the effect of carbonic anhydrase inhibitors on the acid base metabolism of the brain is urgently needed

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The Accumulation of Albumin-^{125}I and Thyroxine-^{131}I in Wounds

By

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Abstract

FUNCH-ROSENBERG, H., H. J. M. HANSEN and E. MOLTKE. *The accumulation of albumin-^{125}I and thyroxine-^{131}I in wounds.* Acta physiol. scand. 1969. 77. 252—256.

The distribution of ^{125}I labelled guinea pig albumin and ^{131}I thyroxine in skin and wounds of guinea pigs has been investigated in the period 1—72 hrs after the simultaneous injection of the radioactive compounds. The relative amounts of labelled thyroxine and albumin in normal skin and skin wounds, old as well as fresh, indicate that these tissues accumulate thyroxine in excess of that brought there on plasma proteins, furthermore that the thyroxine accumulation *increases* in fresh wounds during the experimental period.

During previous work on the inhibitory effect of thyroxine on wound healing many questions as to the mode of action of thyroxine have arisen (Moltke [illegible], 1956, 1957, 1958). It has been shown that the accumulation of endogeneously as well as exogeneously labelled thyroxine is greater in skin wounds than in normal skin (Moltke 1961). It was therefore considered important to examine whether this increase was due to a "passive" leaking-out of thyroxine in the wounds together with other plasma-constituents or was caused by an "active" binding of thyroxine in the wound tissue. The results from this short term experiment (Moltke et al. 1963) suggested that the marked relative retention of thyroxine in skin and 7 days old wounds was not caused by its "passive" presence in the plasma constituents of the tissues. The interpretation was not quite clear as to the accumulation in wounds made at the same time as the radioactive compounds were injected. In these "fresh" wounds a constant plasma-like thyroxine/albumin ratio could best be explained by simple plasma exudation. The results prompted us to extend the duration of the experiment from 24 to 72 hrs in order to reach a period with constant changes of activity.

TABLE I

H p i	Skin		Wounds		Fresh Wounds		Plasma		Gl thyr (total)	
	T	A	T	A	T	A	T	A	T	A
1	9.42	6.49	11.87	12.10	28.73	37.11	278.78	464.87	3.12	1.37
1	8.41	6.03	10.55	9.64	28.89	37.18	155.28	253.39	3.04	1.60
1	10.74	7.57	14.75	14.87		44.48	152.19	257.47	3.38	1.45
Sq	9.5?	6.70	12.39	12.20	28.81	39.59	195.42	325.24	3.18	1.47
6	13.50	9.30	19.80	22.68	29.76	42.64	88.77	174.40	7.63	5.96
6	13.37	7.96	19.62	21.99	33.61	47.51	109.87	192.27	9.05	4.76
6	11.33	6.51	22.66	30.48	39.03	56.79	106.46	193.45	10.63	6.26
Sq	12.73	7.92	20.69	25.05	34.13	49.98	101.70	186.71	9.10	5.66
24	8.96	10.30	16.58	22.89	22.84	39.53	57.56	105.03	24.43	15.18
24	8.78	12.07	14.79	19.02	24.63	35.39	54.05	98.71	23.99	14.75
24	9.42	15.30	13.70	27.79	22.80	44.77	47.12	135.88	17.02	8.17
Sq	9.05	12.56	15.02	23.23	23.42	39.90	52.91	113.21	21.81	12.70
48	5.43	11.07	10.21	21.56	19.45	35.61	30.27	88.19	21.85	11.44
48	4.37	10.88	7.09	17.82	14.84	31.63	24.68	89.65	20.99	11.82
48	5.39	10.93	10.54	19.76	16.67	26.00	29.02	76.37	45.15	24.17
Sq	5.06	10.96	9.28	19.71	16.99	31.08	27.99	84.74	29.33	15.81
72	3.73	11.72	5.23	16.01	10.60	25.86	16.04	79.87	45.35	30.46
73	2.50	9.09	3.79	12.69	8.97	20.71	13.46	62.99	31.98	16.85
72	3.06	9.29	5.14	15.47	13.05	29.21	18.09	78.85	38.00	23.76
Sq	3.10	10.03	4.72	14.72	10.87	25.26	15.86	73.90	38.44	23.69

Pro mille uptake of Thyroxine ^{131}I (T) and albumine ^{125}I (A) in 10 g tissue

Methods

15 male guinea pigs weighing 370—480 g were used all were fed the usual laboratory diet *ad lib*

The animals were given a mixture containing 2 mg ^{125}I guinea pig albumin (= 8 μC) and 1 μg ^{131}I thyroxine (20 μC) the radioactive compounds were injected in one of the jugular veins under ether anesthesia

The guinea pig albumin was prepared for us at the Department of Physical Chemistry Statens Seruminstitut Copenhagen and marked with ^{125}I by us using a modification of the ICl method described by McFarlane the labelled albumin moved electrophoretically like the native albumin ^{131}I thyroxine came from The Radiochemical Centre Amersham

In order to reduce the uptake in the thyroid of radioactive iodide originating from the labelled compounds the animals were all given daily intraperitoneal injections of 0.1 mg stable iodide starting 2 days before the injection of the radioactive compounds

The animals were killed by heart puncture and aspiration of blood under ether anesthesia

Wounds The wounds were made under ether anesthesia as longitudinal linear incisions 1 cm from the dorsal midline the incisions were about 5 cm long and reached the muscular fascia they were closed with continuous silk sutures Two wounds were made on each animal the first wound was made 1 cm to the left the second 1 cm to the right of the dorsal midline

The res[illegible] 1 6 24 48 and 72 hrs after the [illegible] labelled compounds [illegible] old wounds [illegible] old

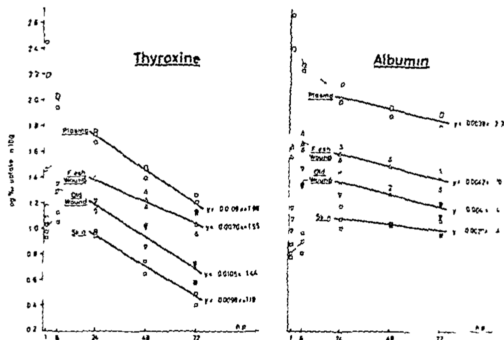


Fig. 1. Residual activity of guinea pig albumin ^{131}I and thyroxine ^{131}I in plasma, fresh wounds, 7 days old wounds and skin. The fully drawn lines are based on regression analysis.

Results and discussion

In Table I the retention of radioactivity in the tissues is expressed as pro mille of injected dose in 10 g. In Fig. 1 the results are reproduced as a function of time in a semilogarithmic system.

It has been shown by others that free ^{131}I iodide is present in blood and tissues only in negligible amounts 24 hrs after the injection of the labelled compounds (Masouredis *et al.* 1953). We can therefore safely assume that all activity found in the blood and tissues is due to the labelled thyroxine and guinea-pig albumin.

Both compounds show highest activity in plasma decreasing through fresh and old wounds and lowest in skin. From 24 hrs after the injection (24 h.p.i.) the decrease follows simple first order kinetics: log(retained) = K + a (h.p.i.). Statistical analysis of the albumin regression lines shows no differences between the 4 examined tissues (mean $a = -0.0063$).

The thyroxine regression lines are alike for plasma, old wounds and skin (mean $a = -0.0101$) but differ significantly ($P < 0.05$) from that of fresh wounds ($a = -0.0070$).

It is most likely that the concentrations of non-labelled albumin and thyroxine are unchanged in plasma and skin during the experimental period. Changes in plasma and skin-radioactivity therefore only reflect changes in specific activity, not changes

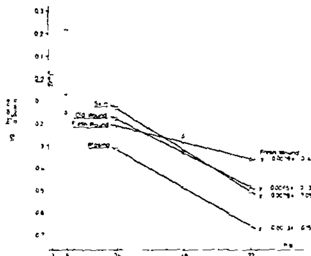


Fig 2 Means of individual thyroxine ^{131}I/albumin ^{125}I ratios in plasma fresh wounds, 7 day old wounds and skin The fully drawn lines are based on regression analysis

in their content of non labelled compounds Although it is known that thyroxine in plasma is bound to globulins (30 %) and prealbumin (46 %) rather than to albumin (20 %) (Hollander *et al* 1962) labelled albumin may still be regarded as a reasonably good indicator for the changing concentrations of high-molecular plasma-constituents in the tissues Because the activity curves from the two types of skin wounds are situated at higher levels than that of skin it is justified to assume that the degree to which the level and regression of the activity curves from these tissues differ from the 'basic" curve of the skin will reflect differences in the amounts of non-labelled substances

The results from the albumin part of the experiment indicate not only that "fresh" wounds accumulate more plasma protein than "old' wounds but also that this accumulation is completed before 24 hrs after the incision Thereafter the turn-over rate does not differ from that of the other tissues Thyroxine however, seems to be accumulated in a different way in the 'fresh' wounds as seen from the less steep regression line for this tissue This difference was tested by an evaluation of the thyroxine/albumin ratio (T/A) for each tissue taken (Fig 2) This T/A gives a more accurate picture of the "real" differences within the single animal, besides the significance of the results was increased because differences due to the different weight of the animals were eliminated Fig 2 shows that T/A is higher in skin and wound tissue than in plasma, probably because of an active peripheral binding of thyroxine like that also seen in many other tissues (Ford *et al* 1957, Tata 1958) The exact parallellism of the T/A slopes for plasma skin and old wounds shows however, that the turn-over in these tissues is fast enough to follow the changing plasma-concentrations For "fresh" wounds the different pattern—already seen when the ^{131}I thyroxine retention was calculated separately—comes out with a much higher degree of significance ($P<0,001$) This indicates that fresh wounds not only con-

tain relatively large amounts of thyroxine but that they take up increasing amounts of plasma free thyroxine during the experimental period. The reason for this is not known, but the increase in the cell mass during the first days after the incision may account for it.

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Acta physiol scand. 1969. 77. 257—260

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Effects of Puromycin and Luteinizing Hormone on the Uptake of 3-O-Methylglucose by the Isolated Rat Ovary

By

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Received 10 January 1969

Abstract

AHRÉN K and L HAMBERGER *Effects of puromycin and luteinizing hormone on the uptake of 3 O methylglucose by the isolated rat ovary* Acta physiol scand 1969 77 257—260

The rate of uptake of the non utilizable monosaccharide 3 *O* methylglucose was studied in

glucose when the protein synthesis was blocked by puromycin

As part of an analysis of the requirements for gonadotrophic stimulation of ovarian carbohydrate metabolism it was recently found that the antibiotic puromycin markedly decreased the rate of glycolysis in isolated ovaries from prepubertal rats (Ahrén, Hamberger and Rubinstein 1968a) It was however still possible to accelerate ovarian glycolysis with gonadotrophins added to the isolated ovaries together with puromycin The mechanism for this inhibitory effect of puromycin on ovarian glycolysis was not further analysed in the abovementioned study but it was suggested that this effect of puromycin might be produced at least in part on the level of the ovarian cell membranes thereby leading to a decreased rate of transport of glucose to the interior of the cells In the present study where prepubertal rat ovaries have been incubated with the non utilizable monosaccharide 3 *O* methylglucose this hypothesis was tested experimentally

It has earlier been reported (Ahrén *et al* 1968b Ahrén Hamberger and Rubinstein 1969) that addition of luteinizing hormone (LH) to isolated ovaries increased the rate of uptake of 3 *O* methylglucose The influence of both puromycin and LH on the rate of uptake of 3 *O* methylglucose was therefore studied in the present experiments

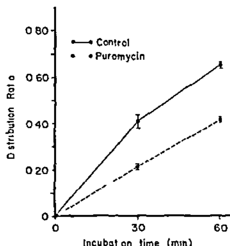


Fig 1 The effect of puromycin on the uptake of 3 *O* methyl ^{14}C glucose by isolated ovaries from prepubertal rats The ovaries were first pre

The uptake of 3 *O* methyl ^{14}C glucose is expressed as distribution ratio (=cpm/ml intracellular water cpm/ml medium) Each value represents the mean of five observations and standard errors are indicated by vertical lines

Methods

Rats of the Sprague Dawley strain, 24—25 days old and ranging in weight between 45 and 55 g, were deprived of food 20—24 hrs and then sacrificed by cervical fracture The ovaries were rapidly removed and trimmed of bursae and extraneous tissue as described previously

on filter paper and first pre one group of ovaries with and the medium The ovaries were onate buffer pH 7 4 0 08 mM dihydrochloride (500 μg/ml) were performed at 37° C with ase Both ovaries from each rat were incubated in each flask (10 ml Erlenmeyer flask with 1 ml medium)

Following incubation the ovaries were removed from the flasks rinsed free of adhering incubation medium by a rapid wash in chilled buffer blotted on filter paper weighed and homogenized in 1 ml trichloroacetic acid (TCA) The radioactivity of the protein free extracts and media was determined in a Packard Tri Carb Liquid Scintillation Spectrometer as described previously (Ahren and Rubinstein 1965)

Total water content of the ovaries was measured by drying the tissue to constant weight the extracellular space was determined by incubating the ovaries with 0 1 mM sucrose U ^{14}C and measuring the percentage distribution of sucrose in the tissue The uptake of 3 *O* methyl glucose was calculated and expressed as the distribution ratio between the intra and extra cellular compartments at the end of the incubation period (cpm/ml of intracellular water cpm/ml medium) It was assumed that the concentration of 3 *O* methylglucose in the extra cellular water at the end of the incubation was the same as in the incubation medium The concentration of 3 *O* methylglucose in the *intracellular water was calculated by subtracting* the amount o determined with sucrose ^{14}C) om the amo water

Bovine LH Study Section of the National Institutes of 1 btained from Nutritional Bio-chemical Corp Cleveland USA and 3 *O* methylglucose and sucrose U ^{14}C from the New England Nuclear Corp Boston USA

Mean values are given ± S E M Comparison between different groups was performed according to Student s t test A p value of 0 05 or less was considered significant

Results

In an initial series of experiments (Fig 1) the influence of puromycin on the transport of 3 *O* methylglucose into the ovaries was studied after 30 and 60 min of incubation This incubation period was preceded by a preincubation period of

TABLE I Effects of puromycin and LH on the uptake of 3 O methylglucose by isolated ovaries from prepubertal rats*

Group	Incubation time min	Addition to medium		Distribution ratio**
		Puromycin 500 μg/ml	LH 100 μg/ml	
1	30	−	−	0 55±0 02 (5)
2	30	+	−	0 35±0 03 (5)
3	30	+	+	0 54±0 03 (5)
4	60	−	−	0 64±0 03 (5)
5	60	+	−	0 40±0 02 (5)
6	60	+	+	0 75±0 01 (5)

* Whole ovaries of prepubertal rats were first preincubated for 30 min in medium without (Groups 1 and 4) or with puromycin 500 μg/ml (Groups 2 3, 5 6) They were then incubated in medium containing 0 03 mM 3 O methyl ^{14}C-glucose with the additions indicated in the table

** Ratio of cpm/ml intracellular water cpm/ml medium Mean±S E Number of observations in parentheses *Significance of differences* 1—2 $p<0\ 001$, 2—3 $p<0\ 005$ 4—5 $p<0\ 001$, 5—6 $p<0\ 001$

30 min in order to create an optimal blocking effect by puromycin on ovarian protein synthesis The results clearly show that the initial rate of uptake of 3 *O*-methylglucose by the ovaries was markedly reduced by puromycin

The second series of experiments was designed to investigate whether LH still could stimulate the transport of methylglucose in the presence of puromycin Thus similar groups of incubated ovaries as mentioned above were again studied, but in this series two groups of ovaries were in addition incubated in the presence of puromycin and LH after preincubation in medium containing puromycin It can be seen from Table I that the ovaries incubated with puromycin (Groups 2 and 5) in this experiment also showed a much slower rate of uptake of methylglucose than the control ovaries (Groups 1 and 4) It can also be seen that addition of LH together with puromycin during the incubation periods (Groups 3 and 6) markedly stimulated the rate of uptake of 3 *O* methylglucose

Discussion

The results of the present study show that incubation of prepubertal rat ovaries in a medium containing puromycin in a concentration which has been shown to block incorporation of labelled amino acids into the ovarian protein with more than 98 % (Ahren and Rubinstein 1965 Ahren *et al* 1968a) markedly inhibits the rate of uptake of the non utilizable monosaccharide 3 *O* methylglucose in the isolated ovaries It has been shown in many other tissues *e g* the muscles *e g* Randle and Morgan 1962) that 3 *O* methylglucose is transported through the cell membranes with th

same carrier system as glucose, and also that this glucose analogue cannot be phosphorylated or in any way metabolized by the cells Previous experiments in this laboratory (Ahren *et al* 1969) have shown that addition of glucose to ovaries incubated with 3 O methylglucose markedly decreased the rate of uptake of 3 O methylglucose, thus indicating that these two hexoses use the same membrane carrier system also in the ovarian cells The result of the present study is therefore a strong argument in favour of the hypothesis suggested in our previous paper (Ahren *et al* 1968a) that the inhibitory effect of puromycin on ovarian glycolysis is produced at least in part on the level of monosaccharide transport through the ovarian cell membranes Further studies are required to elucidate whether this effect on monosaccharide transport is a consequence of an inhibition by puromycin on the synthesis of protein(s) involved in the transport process as has been suggested by others (Kostyo and Redmond 1966, Elsas, Albrecht and Rosenberg 1968) as an explanation for the inhibitory effect of puromycin on amino acid transport in the rat diaphragm

It has previously been reported (Ahren *et al* 1968a, Ahren *et al* 1969) that LH stimulates the rate of uptake of 3 O methylglucose in the isolated prepubertal rat ovary The present study showed that LH still had the same stimulatory effect also when the protein synthesis was blocked by puromycin It is therefore very unlikely that the mechanism for this effect of LH involves an initiation of synthesis of new protein The present results make it more probable that LH, in at least some of the ovarian cells increases the affinity of the membrane carrier for monosaccharides

We wish to thank the Endocrinology Study Section of NIH for the generous supply of LH (NIH LH B5) Most of the vitamins for the semisynthetic diet was a gift from Ferrosan Ltd Malmo Sweden

Valuable technical assistance was given by Mrs Inger Ohlson and Mrs Anita Sjogren

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The Mechanism of the Increased Maximum Work Performance of Small Muscle Groups Resulting from "Diverting Work" with Other Muscle Groups

By

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Abstract

ALPERT, J S *The mechanism of the increased maximum work performance of small muscle groups resulting from 'diverting work" with other muscle groups* Acta physiol scand 1969 77 261—271

The mechanism of increased work performance resulting from diverting works was examined Muscle blood flow was measured in exercised and rested muscles by means of venous occlusion plethysmography and ^{133}Xe clearance Measurements of muscle blood flow blood pressure and work performance following "diverting work and other stimuli were compared with values obtained during control rest periods Stimuli which caused a substantial rise in arterial blood pressure (diverting work", change in heart level pain) caused increases in blood flow and work performance in exercised muscles Non pressor or mild pressor stimuli (non strenuous "diverting work', CO_2 inhalation apnea cold) resulted in unchanged blood flow and work values in the exercised muscles It is concluded that pressor stimuli produce increased muscle blood flow in maximally ischemic muscles due to release of muscle arteries from neurogenic and myogenic control The increased muscle blood flow during periods employing pressor stimuli results in improved muscle restitution and hence increased work performances

In 1914 Weber made a series of observations on the amount of work which a muscle could perform when repeatedly exercised to exhaustion with brief 1—2 min intervals between the maximum efforts The brief pauses were alternately spent resting and performing strenuous exercise with a muscle other than the one which was being repeatedly exhausted Weber noted that his original muscle invariably performed more work after the exercise pauses than after the rest pauses

In succeeding years this observation was enthusiastically employed by Danish gymnastic instructors as means of obtaining better performances from their pupils The instructors referred to the phenomenon as the diverting effect because it was generally felt that the greater work effort performed after exercise pauses was due to distraction of the subject who forgot how tired his maximally exhausted muscle was during these exercise pauses

In 1936 Asmussen observed that the magnitude of the diverting effect varied directly with the size of the muscles and the strenuousness of the exercises employed during the exercise pauses Asmussen believed that the effect was caused by increased

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cardiac output during exercise pauses as compared with complete rest periods He did not, however, present any data to support this contention Recently, Brondum-Laursen (1967) attempted to define more exactly the etiology of the "diverting effect". He was unable to demonstrate any significant change in muscle blood flow associated with the increased work performances which followed exercise pauses

The present study presents data which demonstrates that differences in arterial blood pressure are responsible for the "diverting effect" Increased blood pressure in the intervals with diverting work results in higher blood flow and hence more complete restitution of the muscle during these periods

Definitions

In order to simplify the discussion of the results, the following series of definitions are employed (see Fig 1)

1) The original muscle, repeatedly exercised to exhaustion, is called the standard muscle, its work performance is called standard work, and the period during which it works is termed the standard period

2) The exercise pause is termed the diverting period, and the muscle employed during this period is called the diverting muscle The work performed by the diverting muscle is called diverting work

3) The period of complete rest is termed the rest period A typical protocol used in the present as well as in previous works for eliciting "the diverting effect" is given in Fig 1

Materials and Methods

Subjects Ten normal young men age 25—40 without any symptoms or signs of circulatory, pulmonary or musculoskeletal disease were employed in the study All subjects were volunteers, untrained and unfamiliar with "the diverting effect" Several subjects participated in more than one experiment This was due to the fact that a moderate amount of practice was often necessary to eliminate artifacts from the plethysmographic tracings

... the same extremity by

... is occlusion cuff was

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... ively reported elsewhere (Lassen ... en 1966) In all but a few experi... ically by a Beckman potentiometer ... several consecutive standard diverting and rest periods could ... min ... they ... curve ... ments ... rence ... n two ... artery ... meter

These pressures were recorded by means of a pres...

Blood Pressure Measurements Indirect blood pressures were recorded during numerous ex-

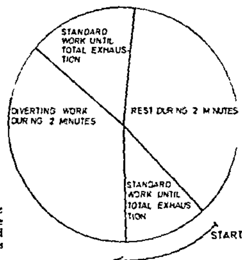


Fig 1 A typical protocol for eliciting "the diverting effect" The experimental procedure always begins with a standard work period and continues until total exhaustion of the tibialis anterior muscle is achieved

Experimental Procedures The right tibialis anterior muscle served as the standard muscle while numerous arm chest and back muscles worked together as the diverting muscle The experiments were performed with the subjects supine on a couch which was firmly attached to the wall An ergograph wi
fixed to the foot of the be
with that part of the leg
belts held both legs and
exercise periods

The right foot was tied securely into a shoe connected to the ergograph. Each time the right foot was fully dorsiflexed the 10 kg weight was lifted 5 cm tripping a microswitch and lighting a small red bulb Dorsiflexions were performed at a rate of 75/min in time with a metronome until total exhaustion of the tibialis anterior muscle was achieved The time required for total exhaustion was noted This enabled one to calculate the amount of work performed during each standard period Rest and diverting periods were 2 min each (see Fig 1) During the rest period the subject was instructed to relax as completely as possible During the diverting period subjects exercised strenuously by swinging a pair of 2 kg hand weights 30 times/min through a 130° vertical arc starting each time from a horizontal position This exercise resulted in approximately 100 kgm of work/min

his tibialis anterior muscle The subject then rested while being supported for the duration of the diverting period Subjects were raised in the prone position by means of a hospital bed scale 2) Pain stimulus—small tufts of hair were plucked from the posterior aspect of the forearm while the subject lay quietly 3 10 % CO_2—the subject inhaled a mixture of 90 % O_2 and 10 % CO_2 while lying quietly 4) Apnea—the subject took as few breaths as possible (usually 2 during a 2 min diverting period) while lying quietly 5 Cold stimulus—the subject had ... ne salt while con-

d in Fig 1 five to
one given experi
ment (see Table No measurements were performed during the first 2 diverting and rest periods of each experiment This was done because the "diverting effect is rarely seen during these first 2 cycles (Weber 1914 Asmussen 1936)

Results

As noted in earlier articles dealing with the 'diverting effect", the amount of work performed after diverting periods was greater than the amount performed after rest periods The present study reports that muscle blood flow measurements using both plethysmographic and tracer clearance methods were greater during diverting periods than during rest periods This was true for diverting periods employing strenuous arm exercise, change in heart level, and pain It was not true for diverting periods employing light exercise without hand-weights, strenuous exercise with the femoral artery compressed, CO_2 inhalation apnea, and cold

Sixteen experiments were performed by 10 different subjects Single average values were calculated in each experiment for the rest period and for the different types of diverting period with respect to the 2 kinds of blood flow measurement and the associated work performances (see Table)

Arm exercises All 16 expts contained diverting periods using strenuous arm exercise with 2 kg hand weights Work performances averaged 54 % greater after these diverting periods than after rest periods Right tibialis anterior muscle (standard muscle) blood flow measured with both techniques was greater during strenuous arm exercise than during rest (^{133}Xe technique 164 % greater, plethysmography 58 % greater see Fig 2 3) All three of these differences were significant ($p < 0.001$)

Work performances and blood flow measurements were essentially identical in rest periods and in diverting periods employing light arm exercises without the 2 kg hand weights (3 expts) This was also the case when the diverting periods contained strenuous arm exercises together with femoral artery compression (3 expts)

Change of heart level Six experiments contained diverting periods employing changes in heart level Work performances were 81 % larger after these diverting periods than after rest periods Standard muscle blood flow as measured by both

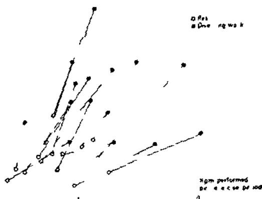


Fig 2 Standard muscle blood flod (m tibialis ant) measured with ^{133}Xe clearance technique during rest and diverting periods (strenuous arm exercises with 2 kg hand weights)

Average values of work performances in kgm/standard period, muscle blood flow in ml/100 g min (^{133}Xe clearance technique), and total calf blood flow in ml/100 ml min (Dohn's air plethysmograph) from 16 expts in 10 subjects Numbers in parentheses in column one refer to the number of experiments in which the subject participated, numbers in parentheses in the remaining columns refer to the number of periods used to calculate each parameter

	Rest			Strenuous work			Change in heart level		
Subject number of experiments No	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min
1	7 4	12 2	7 9	9 9	30 5	10 2	14 2	36 3	11 5
(4)	(32)	(11)	(28)	(18)	(13)	(15)	(3)	(3)	(2)
2	12 0	21 0	10 0	19 1	51 8	15 5	20 3	50 2	16 8
(2)	(12)	(6)	(10)	(10)	(9)	(10)	(3)	(3)	(1)
3	8 9	9 1	6 8	13 9	36 4	8 7	13 2	35 9	14 8
(2)	(12)	(6)	(10)	(9)	(8)	(9)	(2)	(2)	(2)
4	7 0	9 3	4 8	9 5	38 0	10 2	13 0	37 5	12 8
(2)	(10)	(6)	(8)	(5)	(3)	(3)	(1)	(1)	(1)
5	8 4	6 9	6 3	12 4	35 8	11 6			
(1)	(3)	(2)	(3)	(2)	(1)	(2)			
6	8 2	8 5	5 2	13 5	13 0	8 0			
(1)	(3)	(2)	(2)	(2)	(1)	(2)			
7	3 7	11 1	4 0	8 8	18 5	7 5			
(1)	(3)	(2)	(2)	(1)	(1)	(1)			
8	11 2	11 4	4 6	15 0	17 6	6 3			
(1)	(3)	(2)	(3)	(1)	(1)	(1)			
9	7 3	17 8	3 0	11 3	23 9	6 0			
(1)	(3)	(2)	(2)	(1)	(1)	(1)			
10	4 2	6 7	5 8	8 8	15 0	10 0			
(1)	(3)	(2)	(3)	(1)	(1)	(1)			
Average	8 1	11 8	6 4	12 3	31 2	9 9	15 9	41 0	13 7
Total	(84)	(41)	(71)	(50)	(39)	(45)	(9)	(9)	(6)

methods was greater during the change in heart level than during rest (^{133}Xe clearance technique 218 % greater plethysmography 85 % greater) All three of these differences were significant ($p<0\ 01$ $p<0\ 001$ $p<0\ 05$)

Pain stimulus Five experiments contained diverting periods employing a pain stimulus Work performances after these diverting periods were increased 57 % as compared with performances following rest periods The blood flow in the standard muscle was seen by both measuring techniques to be greater during the diverting periods than during the rest pe[illegible] nique 171 % at

Subject number of experiments No	Pain			Femoral artery compression			Non strenuous work		
	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min
1	11 8	38 0	10 0	7 6	14 6	6 0	6 9	13 1	7 6
(4)	(2)	(2)	(2)	(3)	(3)	(3)	(1)	(1)	(1)
2	18 9	43 2	17 3						
(2)	(1)	(1)	(1)						
3	15 7	33 4	11 4						
(2)	(2)	(1)	(2)						
4									
(2)									
5									
(1)									
6							6 5	9 1	5 8
(1)							(1)	(1)	(1)
7							9 0	16 5	7 1
(1)							(1)	(1)	(1)
8									
(1)									
9				7 1	16 2	5 3			
(1)				(2)	(2)	(2)			
10				5 2	7 5	4 8			
(1)				(3)	(3)	(3)			
Average	14 8	38 2	12 4	6 6	12 3	5 4	7 5	12 9	6 8
Total	(5)	(4)	(5)	(8)	(8)	(8)	(3)	(3)	(3)

SKIN AND MUSCLE BLOOD FLOW

(PLETHYSMOGRAF)

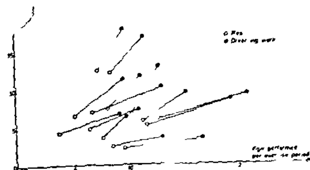


Fig 3 Total calf blood flow, recorded by Dohn's air plethysmograph during rest and diverting periods (strenuous arm exercises with 2 kg hand weights)

Subject number of experiments No	Cold			Apnea			CO_2		
	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min	Work performance Kgm	Muscle blood flow (^{133}Xe technique) ml/100 g min	Total calf blood flow (plethysmography) ml/100 ml min
1 (4)	8 2 (3)	11 9 (3)	8 3 (3)	7 9 (2)	20 4 (2)	8 2 (2)	7 7 (2)	12 6 (2)	7 1 (2)
2 (2)									
3 (2)				9 3 (1)	35 1 (1)	7 4 (1)			
4 (2)									
5 (1)							8 0 (2)	7 4 (2)	6 0 (2)
6 (1)									
7 (1)									
8 (1)				10 8 (2)	11 7 (2)	5 0 (2)			
9 (1)									
10 (1)									
Average Total	8 2 (3)	11 9 (3)	8 3 (3)	9 3 (5)	19 9 (5)	6 7 (5)	7 9 (4)	10 0 (4)	6 6 (4)

plethysmography 51 % greater) All three of these differences were significant ($p<0.025$, $p<0.005$, $p<0.02$)

CO_2 inhalation, apnea and cold stimulus These three stimuli were used in 2, 3, and 1 expt respectively No consistent differences were seen in either blood flow measurements or work performances between rest periods and diverting periods using these stimuli

Blood flow in standard periods As mentioned above, plethysmographic records could not be obtained during standard periods due to artifacts caused by the contracting tibialis anterior muscle ^{133}Xe clearance curves were, however recorded during standard periods in all 16 experiments These curves demonstrated clearly that no blood flow occurred during these periods This observation was supported by clinical impressions in that all experimental subjects described typical ischemic pains in the right tibialis anterior muscle during the standard period Mor

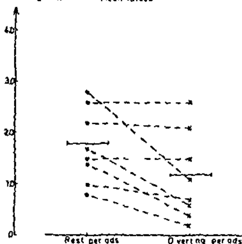


Fig 4 Blood flow in resting muscles measured with ^{133}Xe clearance technique during rest and diverting periods (strenuous arm exercises with 2 kg hand weights)

that the subjects could work for only 20—40 sec during each standard period also argued for a cessation of blood flow in the standard muscle during these periods

Blood flow in resting muscles Nine experiments were performed to investigate the effect of strenuous arm exercise on blood flow in the resting *left* tibialis anterior muscle (6 expts) and the resting *right* gastrocnemius muscle (3 expts) Plethysmographic recordings of the resting blood flow could only be obtained from the left leg while ^{133}Xe clearance studies could be performed selectively in the resting muscles of both legs ^{133}Xe clearance curves were recorded simultaneously in these experiments by a logarithmic potentiometer writer and by a digital ratemeter coupled to a print out recorder The values obtained from the print out recorder were plotted on semi log paper and the curve was compared with that obtained using the logarithmic potentiometer writer The 2 curves from any single experiment were essentially identical

ood flow in the resting muscles measured by ^{133}Xe clearance decreased by 44 % ıg the diverting periods as compared with the rest periods (see Fig 4) This ference was of borderline significance ($0.05 < p < 0.1$) Total calf blood flow quals essentially skin + muscle blood flow) measured by the venous occlusion plethysmograph increased by 18 % in the resting leg during the diverting periods (Fig 4) This difference was significant ($p < 0.05$) and can only be explained by a substantial rise in skin blood flow during the diverting periods

Blood pressure measurements The results of the indirect and direct blood pressure measurements were invariably similar During diverting periods employing strenuous work, change in heart level and pain higher blood pressure values were obtained than during rest periods and diverting periods employing non strenuous work CO inhalation, apnea and cold Mean and diastolic blood pressures averaged 25—40 mm

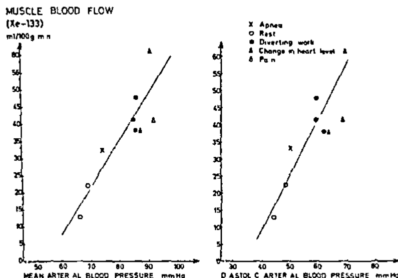


Fig 5 Results of a typical experiment with direct blood pressure measurements in the femoral artery The blood flow in the standard muscle measured with the ^{133}Xe clearance technique is compared with mean arterial and diastolic blood pressure during rest and different kinds of diverting periods

Hg greater during diverting periods with pressor stimuli than during rest periods or diverting periods with non pressor stimuli The results of a typical experiment are seen in Fig 5 (directly recorded blood pressure)

Discussion

The present series of experiments demonstrates that the diverting effect is dependent upon increased arterial blood pressure during diverting periods Intramuscular tension during standard work undoubtedly exceeds the perfusing blood pressure of the standard muscle because blood flow at this time essentially ceases (Barcroft and Millen 1940 Nilsson and Ingvar 1967) Standard work is carried out until the ischemic conditions in the muscle prevent further contractions Restitution of the muscle occurs during succeeding rest and diverting periods This restitution is directly dependent upon blood flow during these periods which is in turn dependent upon the driving blood pressure

Stimuli which increase arterial blood pressure (exercise change of heart level pain) were found to result in higher standard muscle blood flow This relationship between arterial blood pressure and muscle blood flow has been previously noted by Lind and McNicol who studied muscle blood flow during static work of varying intensity (Lind and McNicol 1967) As mentioned above Brondum Laursen was unable to demonstrate any significant change in muscle blood flow during diverting work periods Brondum Laursen employed three minute rest and diverting periods in his experiments and his results demonstrate that muscle blood flow at the end of

these three minute periods had essentially returned to the basal resting value Since the standard muscle was almost completely repaid after each three minute rest or diverting period it is not surprising that blood flow values for these two types of period did not differ significantly

The beneficial effect of the pressor stimuli on muscle restitution can be blocked by compromising the femoral artery with external digital compression If the stimuli are presented in a diluted form i e non strenuous exercise, the pressor effect is greatly reduced or absent causing flow values and muscle restitution of the same order of magnitude as during rest periods Non pressor stimuli (CO inhalation apnea and cold) gave no diverting effect The fact that CO inhalation apnea and cold were found to cause inconsistent flow and pressor responses confirms earlier reports (Boyer Fraser and Doyle 1960 Sechzer *et al* 1960 Raper *et al* 1967)

Blood flow in resting muscle showed changes which were the opposite of those seen in the standard muscle flow values were higher during rest than during diverting periods This finding exactly parallels the results of Blair Glover and Roddie (1961) These investigators found a decrease in resting muscle blood flow during exercise resulting from an increase in sympathetic vasoconstrictor tone

It is generally agreed that arteries are subject to two different types of control neurogenic (autonomic nervous system) and intrinsic (probably myogenic) Numerous investigators have shown that a neurogenic vasoconstriction occurs during strenuous exercise in diverse organ systems including resting skeletal muscle and skin (Barger *et al* 1949 Bishop *et al* 1957 Muth *et al* 1958)

Intrinsic (probably myogenic) factors in the wall of the arteries themselves oppose flow changes when perfusing pressure is altered as it is during exercise This is the well known phenomenon of autoregulation (Jones and Berne 1964 Stainsby 1964)

Both neurogenic and intrinsic control factors are responsible for the reduced or unchanged blood flow seen in the resting muscles during strenuous exercise Conversely standard muscle blood flow increased during diverting periods employing arm exercise This could only occur if the arteries had been released from the influence of autoregulation and vasoconstricting sympathetic tone That such a release *is brought about by ischemia or high concentrations of metabolites had been amply* demonstrated (Lewis and Mellander 1962 Remensnyder Mitchell and Sarnoff 62 Kjellmer 1965 This then is the explanation for the increased bloow flow seen the standard muscle during diverting periods with pressor stimuli the increased terial blood pressure is able to cause increased blood flow in the standard muscle because the ischemia or metabolites produced during standard work releases the muscles arteries from intrinsic as well as neurogenic control The vessels dilate and behave essentially as a rigid hydraulic system in which changes in perfusing pressure result in changes in flow

Skin arteries do not exhibit autoregulation and their control is essentially only neurogenic Skin flow was indirectly seen to increase during diverting periods employing arm exercise This may be a reflection of the passive nature of skin vessels which carry more blood when the perfusing pressure increases (Barger *et al* 1949)

The increase in skin flow which paralleled the increase in arterial blood pressure may also have been due to release of skin vessels from sympathetic vasoconstrictor tone because of the increased body temperature resulting from the strenuous arm and leg exercises Indeed, all subjects were seen to perspire during the experiments

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Nature and Localization of the Sodium Pool during Active Transport in the Isolated Frog Skin

By

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Abstract

Zerahn, K. *Nature and localization of the sodium pool during active transport in the isolated frog skin.* Acta physiol. scand. 1969. 77. 272—281.

In the frog skin epithelium the amount of radio-sodium originating from the outside solution has been determined under different experimental conditions. *It has been found that this sodium pool behaves largely as a pool in which the sodium has passed the transport mechanism.* It has not been possible under any of the conditions used to demonstrate the existence of epithelial radio-sodium awaiting active transport. This finding leads to the conclusion that the mechanism for active transport of sodium is effective at the outer surface of the frog skin.

The isolated frog skin actively transports sodium from the outside to the inside. By labelling the sodium on the outside and awaiting constant influx, radio-sodium can be assumed to have attained a steady state in the skin. The labelled sodium will then be in all phases normally present during active transport. One fraction may be awaiting transport (transport pool W, Fig. 1), another fraction already transported (P) will be diffusing out of the skin to the inside solution. Finally, some of the labelled sodium may be diverted from the direct transport path into some compartments, sodium thus being retarded on its way to the inside solution.

On quickly removing the outside solution the amount of labelled sodium in the in can be determined and the rate of disappearance can be followed. The content labelled sodium is determined by placing a G M counter over the skin after the outside solution has been removed. The disappearance rate of radio-sodium to the inside solution is determined by measuring the radio activity of the skin with time (Andersen and Zerahn 1963).

In a semilogarithmic plot of radio-activity against time the curve can be resolved into two straight lines and the corresponding half times determined. One has a slope with a half time of a few minutes, the other about 30 min. The slow component contains at time zero usually around 10 % of the activity, the fast component the bulk of the labelled sodium. The intercept of the fast component with the ordinate

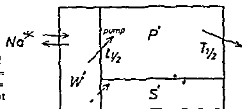


Fig 1 W' = compartment for transport pool W'. $t_{½}$ = disappearance half time for W'. P' = compartment for transported pool P'. $T_{½}$ = disappearance half time for P'. S' = compartment for secondary pool S' (part of slow component)

is taken to give the steady state radio-sodium pool (called Na* pool) through which transepithelial sodium transport takes place

In as much as it has never been possible to resolve the curve for the disappearance rate of radio-sodium into more than two straight lines the Na* pool could be either an epithelial sodium pool awaiting transport (W') or an already transported sodium pool (P)

In the model used (see Fig 1) the compartment W' for the sodium awaiting transport should have the properties of a layer of cells with a kind of Gibbs Donnan equilibrium with a sodium pump towards the inside With the boundaries permeable to several ions one would expect

An inhibitor with an effect on the pump only, should cause W' to increase and the rate at which sodium leaves W' to decrease However as yet there is no agent known which has a proven inhibitory or stimulative effect directly and exclusively on the pump The following inhibitors for sodium transport were however used low pH dinitrophenol ouabain and procaine As stimulators were used vasopressin on the inside of skins from brown frogs (*Rana temporaria*) and procaine on the outside of skins from green frogs (*Rana esculenta*)

Of a transported pool one would expect that

1) An inhibition of the pump would decrease P but leave the rate of disappearance of radio-sodium from the pool by diffusion unchanged

2) A stimulation of the pump would increase P and not influence the disappearance rate

Following these considerations is has been shown within the limitations of the method used that the Na* pool behaves as if it has passed the transport mechanism and all attempts to find conditions where a transport pool could be demonstrated have been in vain This finding disagrees with the opinions expressed in the earlier paper (Andersen and Zerahn 1963) The assumption generally accepted at that time that the transport mechanism is at the inside border of the epithelium is shown by the present work to be incorrect

Methods

Pool determination

The methods for determining Na* pool has been described in the earlier paper *l c* the standard deviation for several determinations of the Na* pool on the same skin was ±7 % For the half time the S D was ±0 2 min for measurements on the same skin at room temperature under the same experimental conditions

The so called graphical pool method also described in the earlier paper was used in those cases where the slow component is increased so much in size that the determination of Na* pool was impossible Table 2 shows good agreement between half times calculated by the two methods in the control periods

Sectioning of skin

The method described by Hvid Hansen and Zerahn (1964) has been used The principle is to place the skin on a cold agar plate cut plane with the knife from the freezing microtome The epithelial side was facing downwards and when a good tight contact was obtained by spreading the skin the skin was frozen to the agar The sectioning was performed in the cold room because this minimises the disturbance by condensing water vapours

Extracellular space

The extracellular space was determined by labelling the inside solution 10 % of the chloride in Ringers was substituted by an equivalent amount of sulphate labelled with S 35 The apparatus used was similar to the one used for pool determination but with a volume of inside solution of only 5 or 15 ml With distilled water on the outside the Na* pool is zero and the total amount of sodium in the outermost layer of the skin is as low as possible When equilibrium with respect to S 35 in the epithelium was reached the skin was removed cut out and sectioned parallel to the surface as described by Hvid Hansen and Zerahn (1964) The sections were placed in small covered quartz beakers weighed and dissolved in nitric acid by heating for 20 hrs at 90° C Then the acid was allowed to evaporate and 3 ml of distilled

section

Solutions

Na Ringers (Na R) NaCl 115 mM $KHCO_3$ 2 5 mM $CaCl_2$ 1 0 mM
Mg Ringers (Mg R) $MgCl_2$ 57 5 mM $KHCO_3$ 2 5 mM $CaCl_2$ 1 0 mM

Results

a) *Stimulators of active sodium transport* The results obtained with insipidin administered to the inside solution and with procaine administered to the outside solution are shown in Table I There was an increase in the Na* pool but no significant alteration in the half time

b) *Inhibitors of active sodium transport* (I) Low pH dinitrophenol and procaine decreased the Na* pool in proportion to the decrease in active sodium transport but again there was no change in the half time (Table I)

(II) Ouabain has generally been considered an inhibitor of active transport (for references see Ussing 1960) Administered to frog skin it causes a decrease in active sodium transport The logarithmic curves for determination of the Na*-pool become one straight line only (Fig 2) At the same time the total amount of labelled sodium in the skin increases from around 0 5 μeq to as much as 3 μeq with a disappearance half time of as much as 40 min Since the disappearance rate for the Na* pool can not be found with the present method another approach was used namely the graphical method (Andersen and Zerahn 1963) The time lag obtained by extrapolating the flux curve (Fig 3) from steady state back towards the intersection with the abscissa is twice the half times Table II summarises the result In control periods the half times found with the usual method and the graphical method agree well Upon administration of ouabain the graphical half time is somewhat lengthened but

TABLE I Effect of stimulating and inhibiting sodium influx on the Na* pool and half time in the short circuited skin of *Rana temporaria* and *esculenta** Area 7 cm² Room temperature Bathing solutions Ringer s Each figure is a mean value of 3—5 expts Sodium influx is computed from pool and half time

	pool μeq Na		half time min		Na influx μeq hr	
	contr	exp	contr	exp	contr	exp
Stimulation						
Pituitary hormone (ADH) inside 0 17 i u /ml	0 63	1 06	3 0	2 4	8 7	18 3
*Procaine outside 11mM	0 45	0 70	3 8	3 8	4 9	7 7
Inhibition						
pH 8 0 6 0	0 74	0 33	3 3	3 7	9 3	3 7
DNP 3 × 10⁻⁴M	1 73	0 60	2 1	2 1	34 2	11 8
*Procaine inside 11 mM	0 52	0 23	3 6	4 1	6 0	2 3

much shorter than the half time for the curve from the ouabain experiment in Fig 2

c) *Influence of sodium concentration* Table III shows the effect of lowering the concentration of sodium in the outside solution It can be seen that the Na* pool decreases with a decrease in sodium concentration However even at very low out-

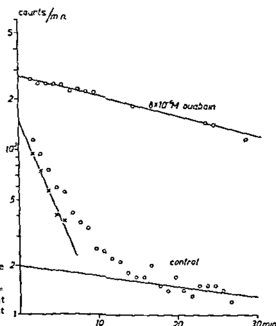


Fig 2 Curves for the disappearance of labelled sodium from frog skin
2580 cpm = 1 15 μeq of sodium = 10 μl outside solution The straight lines are drawn by the method of least squares

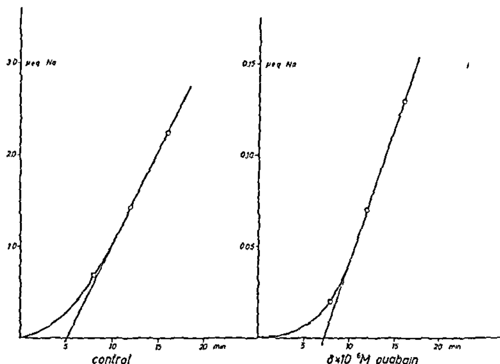


Fig 3 Curves for obtaining half times by the graphical method The curves in Fig 2 and Fig 3 are obtained from the same skin

TABLE II Effect of ouabain on the short circuited skin of *Rana temporaria* Inside and outside Ringer's solution Area 7 cm²

ouabain conc µM	Na* half time min	graphical half time min	Na influx at steady state µeq/hr	current µeq/hr
0	2 8	2 5	12 2	11 2
8	—	3 7	0 9	0 9
0	2 5	1 8	9 2	9 4
0 8	—	3 2	2 5	1 6
0	2 3	2 5	12.6	12 0
1 3	—	4 0	1 2	1 2
0	2 2	2 5	6 7	6 5
0 5	—	3 2	2 2	1 6
0	3 4	2 7	10 3	9 9
0 8	—	3 5	2 3	2 0
mean value	control	2 4		
mean value	ouabain	3 5		

TABLE III Relation between the sodium concentration in the outside solution and the Na* pool in short-circuited skins of *Rana temporaria* Area 7 cm² Room temperature The low sodium concentrations are obtained by diluting the Na R with Mg R

Na outside µeq/ml	pool µeq Na	half time min
115	0.67	2.6
11.5	0.40	2.1
2.3	0.11	2.6
115	0.99	4.7
11.5	0.31	4.2
2.3	0.11	4.0
115	1.04	1.8
2.3	0.052	1.7
0.32	0.022	1.8
115	0.92	1.5
2.3	0.19	1.2
0.32	0.06	1.6
115	1.35	2.2
2.3	0.23	1.5
0.23	0.043	1.7

side sodium concentrations the logarithmic curves can be resolved into two straight lines only and the half-time is unchanged No significant change in the size of the slow component was observed The low sodium concentrations of the outside solution were confirmed by flame photometry at the end of the incubation period

d) *Influence of low temperature* It has been found that at temperatures just above 0° C the active sodium transport is less than at room temperature The pool is not decreased (in fact, it may be slightly increased) but the disappearance rate of labelled sodium from the pool is decreased to give a mean half time of around 15 min (Table IV) Whether the Na*-pool was a transported pool or was awaiting transport was tested This was done as outlined in experiments at room temperature and with similar results

Cellular sodium in epithelial sections of isolated skin

The best estimate of cellular sodium concentration was obtained by subtracting the amount of extracellular sodium from the total amount of epithelial sodium The results are listed in Table V It can be seen that the cellular concentration of sodium in the outermost layer of the epithelium is 18 mmolar and does not vary much whether the section is light or heavy *i e* consists of just one or more cell layers There is thus no indication of a cell layer with a very low sodium concentration The estimate of cellular sodium concentration most likely is too low as the outer sections contain cornified cells and probably some agar free of sodium If the amount of sodium in the epithelial sections are corrected to the same potassium concentration, the two outermost layers show an almost equal sodium concentration in the cells

TABLE IV Effect of temperature on Na* pool in short circuited skins from *Rana temporaria* Area 7 cm²

	temperature °C	pool µeq Na	half time min
first day	5 0	1 29	19
second day	3 5	0 78	20 5
	23 6	0 76	2 6
first day	22 8	0 63	2 2
	2 5	0 88	12
second day	2 5	0 58	11 5
	23 2	0 58	2 8
first day	23 2	0 78	2 6
	3 8	1 38	22
second day	2 5	0 46	14
	21 5	0 52	2 2

TABLE V Extracellular space in the epithelium of the isolated skin of *Rana temporaria* and concentration of sodium and potassium in the epithelial cells at different distances from outside computed from the weight and the area of 6 6 cm² sections [Na] at constant [K] computed from [Na]/[K] times [K] in second section

wet weight mg	% extracell space	Na conc mM	K conc mM	[Na] at constant [K]	thickness of section in µ from outside
5 1	14	13	78	21	0—7
10 4	13	26	125	26	7—21
22 9	29	33	133	31	21—53
9 5	10	18	102	24	0—13
10 5	19	46	138	46	13—27
20 7	43	30	133	31	27—56
5 5	11	14	60	27	0—8
10 5	11	24	117	24	8—22
18 8	31	29	140	24	22—48
3 6	8	25	50	49	0—5
8 1	11	44	98	44	5—16
15 0	29	40	134	30	16—37
outer section	11	18 mean values			
inner section	14	35 mean values			

Discussion

Fig 1 shows in a schematic way that the sodium from the outside of the skin first must pass a transport pool W and afterwards by action of the sodium pump reach the transported pool P located for example in intercellular spaces In a steady state the active sodium transport through the skin must be equal to the flux through W and to the flux through P Assuming that disappearance from the pools can be described by straight lines on a semilogarithmic plot, the following formula must be valid

$$\text{sodium influx } \mu eq/hr = W \times 0\ 69 \times 60/t_1 = P \times 0\ 69 \times 60/T_1 \qquad (1)$$

where the half times are given in minutes the pools in μeq and the flux in μeq/hr

As mentioned an inhibition of the pump should increase t_1 and increase W at the same time decreasing P without any effect on $T_{\frac{1}{2}}$ provided ions other than sodium can pass into and out of compartment W If the Na* pool were mainly a transport pool inhibition of the sodium pump should result in an increase of both pool and half time However it can be seen from Table I and III that the pool decreases whereas the half time is unchanged The reduction of the pool by the action of inhibitors such as low pH or procaine could be interpreted as a tightening of the outside border of W for sodium and not as an inhibition of the pump However the action of DNP cannot be considered to leave the pump unchanged in giant axons DNP (Hodgkins and Keynes 1955) inhibits the active extrusion of sodium and *apparently does not effect the passive movement of sodium across the cell membrane* The effect is probably via the metabolism as ATP or arginine phosphate temporarily restores active extrusion of sodium in inhibited axons It is thus reasonable to assume that DNP inhibits the transport mechanism in the frog skin whereas there is no reason for assuming a change of permeability of the outer membrane of W DNP penetrates easily into the cell and will interfere with the common source of energy for both transport and non transport processes The inhibition is known to take place at low inhibitor concentration by uncoupling oxidative phosphorylation At higher concentrations other effects appear besides uncoupling the slow component of the curves increases *very much at inhibitor concentrations of* 10^{-4} M or higher Experiments with cyanide gave the same result The half time is unchanged and Na* pool decreases with the current Thus we have to conclude that the Na* pool is mainly a transported pool under these conditions

The sodium concentration in the outermost epithelial cells was found to be 18 mM in the mean when the outside solution was distilled water Table V The values can be expected to be higher during active sodium transport The measurements could be considered doubtful from the point of view that the sodium could be derived by movements in the frozen state from the sodium in the innermost layers The extracellular space determined with labelled sulphate is however in the outer layer 11 % and in the next 14 % but in the rest of the epithelium shown in Table V the mean value was 33 % The intra[illegible]
mM in the next 35 mM an

extracellular space cannot move in the frozen state without dragging the sulphate along so diffusion in the frozen state is unlikely.

With 18 mM sodium or more in the cells the passive movements from the outside sodium concentration of 0.2—0.3 mM demands a potential difference of around 120 mV (cell negative) to obtain the same electrochemical potential in the cell and outside solution. The potential difference (50 mV) measured by Ussing and Windhager (1964) can not give the information because their measurements are on the cells from the middle of the epithelium. The assumption that a transport mechanism is located at the inside cell wall of the first epithelial layer would include that the sodium in this layer should be identical with the transport pool. The values of Na^* pool shown in Table III are however lower than the sodium content in the outer cell layer and we know furthermore that the Na^* pool is located over the whole epithelium.

The 18 mM value is calculated with the assumption that the sodium is distributed evenly in the cellular water. However, there may be some places where the concentration is higher, like the nucleus. If this shall decrease the sodium in the cytoplasm significantly the concentration in the nucleus must be very high.

The Na^* pool can not be determined after the addition of ouabain as mentioned in results. The graphical method used for measuring sodium pool half time has no assumptions of the route of the sodium through the skin but gives the time lag until the steady state is obtained. By dividing this time lag by 2 we obtained an empirical half time (Fig. 3).

Referring to Fig. 1 it is apparent that if the radio sodium passing by the secondary pool compartment S exchanges slowly with unlabelled sodium in S the graphical method and the direct pool measurement will give almost identical half times. This is actually found in control experiments (Table II). However during ouabain treatment the usual method shows the skin to contain as much as 3 μeq of sodium taken up from the outside with a long half time whereas the graphical half time is only slightly longer despite the fact that the current is reduced and that the total amount of radio-sodium in the skin is increased. This must mean that with ouabain treatment the actively transported sodium which is responsible for the short circuit current passes through a transport pool as small or smaller than that present in the control periods. The major part of radio-sodium accumulated in the skin during ouabain inhibition thus has no direct connection with transepithelial sodium transport. The site of this Na must be in the cells as this is the only place where 1 to 3 μeq of sodium can be retained. This is in agreement with the observation that ouabain causes up to 5 μeq of potassium to be lost by the 7 cm^2 skin depending on the degree of inhibition (Koefoed Johnsen personal communication).

How do these findings agree with earlier data and views on the sodium transport of the skin? The work of Farquhar and Palade (1964) and of Takenaka (1963) shows that there is an intercellular space in the epithelium of the frog skin. This may be a likely place for Na^* pool. From a 30 μ thick layer of Ringer's solution one would expect a diffusion time of a few seconds for 90 % equilibration (Jacobs 1935)

However, intercellular space constitutes a devious pathway for diffusion longer than 50 μ Furthermore, the cross sectional area of intercellular space varies through the epithelium Finally the fractional area open for diffusion at the basement membrane may be small

Conclusion

The Na* pool behaves as if the labelled sodium has pasesd the transport mechanism This interpretation disagrees with opinions expressed in the earlier paper (Andersen and Zerahn 1963) The concept that the Na* pool is a transport pool was based on the wrong assumption that the transport mechanism was at the inside border of the epithelium However there must be some sodium awaiting transport or being in the process of transport This amount has to be a very small fraction of the Na* pool and consequently have a very short half time therefore detection will be difficult A 50 μ thick layer of cells or even a one cell thick layer of cells with a sodium content of 20 or 30 mM is not suited as a transport pool

The conclusion is that sodium is transported in such a way that it does not mix immediately with cellular sodium It is not yet clear whether or not the transport takes place through the cells via some structure taken in the broadest sense or along the cell membranes between the cells neither is it known how far the mechanism reaches through the epithelium

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Permeability Characteristics of Vascular Smooth Muscle Cells as Revealed by Their Osmotic Responses to Non-Electrolytes

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Abstract

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Changes of cell volume in the smooth muscle of the isolated rat portal vein produced by variations in extracellular osmolarity are associated with characteristic changes in the spontaneous electrical and mechanical activity of the preparation (Johansson and Jonsson 1968, Arvill, Johansson and Jonsson 1969). An analysis of such responses to anisosmotic solutions containing different non-electrolytes was carried out in the present study in order to elucidate the osmotic influences of the test substances on the vascular smooth muscle cells. The time course of the responses revealed the following relative rates of penetration through the cell membrane: sucrose < erythritol < glycerol < urea < thiourea < ethylene glycol = propylene glycol = tetramethylene glycol = pentamethylene glycol = urethane. The importance of molecular size and lipid solubility for this order of permeability is discussed. A quantitative analysis of responses to solutions containing urea gave an approximate estimation of the osmotic reflexion coefficient for this substance. The value obtained was 0.65 which corresponds to an equivalent pore radius of 3.7 Å. This information and previous data concerning the uptake of urea-^{14}C by the rat portal vein could be used for a calculation of the total pore area. The result indicated that the pores occupy only a very small fraction (7×10^{-7}) of the total area of the cell membrane and that the number of pores is of the order of 10^3 per cell or 1.6×10^{8} per cm^2.

The smooth muscle of the rat portal vein is characterized by a consistent and quite regular spontaneous contractile activity when studied *in vitro* in a standard Krebs solution (Funaki and Bohr 1964). Recent experiments have shown that changes in the cell volume of this vascular smooth muscle induced by variations in extracellular osmolarity are accompanied by typical changes in the spontaneous activity (Johansson and Jonsson 1968, Arvill, Johansson and Jonsson 1969, Jonsson 1969). This preparation thus showed a consistent relationship between cell volume and muscle activity, shrinkage being associated with inhibition, swelling with excitation. For instance, an increase in the osmolarity of the extracellular fluid, produced by addition of sucrose to the standard Krebs solution, led to shrinkage of the smooth

muscle cells and sustained inhibition of their electrical and mechanical activity. Solutions made hyperosmotic by the addition of urea produced electrical and mechanical responses of short duration which could be attributed to the transient alterations in cell volume obtained with this permeant substance. Hypotonic solutions caused swelling of the cells, depolarization and sustained increase in phasic activity.

Since changes in the volume of these vascular smooth muscle cells are thus reflected in their pattern of activity it seemed possible to study the osmotic influence of different non electrolytes on the cell volume by merely observing the mechanical responses of the muscle to anisotonic solutions containing such substances. This in turn would provide information about the permeability characteristics of the cell membranes. The present report describes responses of the portal vein to various non electrolytes of different molecular weights and lipid solubilities. Some quantitative calculations regarding the membrane permeability of the smooth muscle cells have been performed.

Methods

The experiments were performed on Sprague Dawley rats with body weights varying between 200 and 350 g. The animals were fed a commercial laboratory diet and they had free access to
n long were dissected free immediately
The vessel was cut open longitudinally
rgan bath containing Krebs solution of

the actual experiment was started. The preparations maintained their spontaneous rhythmic activity and gave reproducible responses to variations in tonicity for several hours under these experimental conditions.

Hyperosmotic solutions containing different non electrolytes were prepared by adding the respective substances to the standard Krebs solution. Solutions with decreased tonicity were obtained by reducing the NaCl concentration of the Krebs solution from 122 mM to 92 or 102 mM. The osmolarity of such "NaCl low" solutions was restored (or increased above normal) by the addition of equiosmolar (or greater) amounts of the different test substances. Measurements of freezing point depression (Osmometer 31 LAS, Advanced Instruments Inc.) were made on the different solutions to check their total osmolarity. The experimental variations of the medium described in Results below will be presented mainly in terms of changes in molar concentrations. Table I gives the relationships between molar concentration and osmolarity
tab

the
tion reached the muscle preparation has consistently been marked as the beginning or the end of the exposure periods.

Experiments were performed on a total of 52 portal vein preparations.

Results

I Substitution of different non electrolytes for NaCl

Previous experiments with the isolated portal vein showed that a decrease in the tonicity of the Krebs solution by reduction of its NaCl concentration led to de lari

TABLE I

Substance	Mol weight	mOsm / mmoles	Oil/water partition coefficients x 10³ (Collander 1954 Davson 1964 p 286)	Mol radius Å from Goldstein and Solomon (1960)* or calculated from mol weight and density
Sucrose	342	1 03	0 03	4 40
Erythritol	122	1 01	0 03	3 06*
Glycerol	92	0 99	0 07	2 74*
Urea	60	0 99	0 15	2 03*
Thiourea	76	0 93	1 2	2 18*
Ethylene glycol	62	1 00	0 49	2 24*
Propylene glycol (1 2 Propanediol)	76	1 00	1 7	2 74*
Tetramethylene glycol (1 4 Butanediol)	90	0 98	2 1	3 28
Pentamethylene glycol (1 5 Pentanediol)	104	0 97	6 1	3 46
Urethane	89	0 99	74	3 30
Sodium chloride	58 5	1 83		

zation and sustained increase in contraction frequency (Johansson and Jonsson 1968) This excitatory response did not occur if the removal of NaCl was compensated for osmotically by addition of an equiosmolar amount of sucrose Specific effects of the decrease in [Na] or [Cl], are thus of negligible importance as long as the changes in these ionic concentrations are moderate

A series of experiments has been performed in this study in order to evaluate the ability of different organic substances to act as osmotic substitutes for NaCl with regard to the effect on the vascular smooth muscle Fig 1 illustrates a series of recordings from such an experiment The typical spontaneous rhythmicity of the portal vein characterized by phasic contractions at a frequency of 3 to 5 per min is seen in the control periods preceding the exposures to the test solutions In Fig 1A the muscle was exposed to a solution in which the NaCl concentration was re ced by 20 mmoles/l without any osmotic substitution As described in earlier ports there is an immediate and sustained increase in mechanical activity Fig 1B ws that an excitatory response does not occur if isotonicity is maintained by addition of sucrose which again is in agreement with our earlier findings (Johansson and Jonsson 1968

Urea enters the smooth muscle cells and equilibrates with the total tissue water of the portal vein preparation in about 10 to 15 min (Arvill et al 1969) Therefore if used as an osmotic substitute for NaCl in the medium it should not prevent the cells from swelling As expected there is a clear-cut excitatory response in Fig 1C but this develops more gradually than in Fig 1A This slow increase in muscle activity is considered to reflect a slow swelling of the cells which in turn is attributed to the restricted rate of penetration of urea into the intracellular space

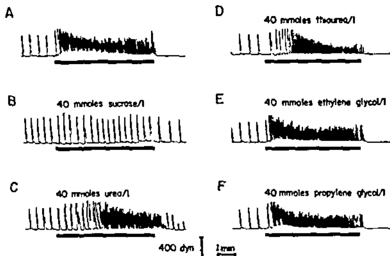


Fig 1 Mechanical responses of a portal vein to a hypoosmotic solution (A) and to solutions in which different non electrolytes were used as osmotic substitutes for NaCl (B—F)

Thiourea has a larger molecular weight than urea (Table I) and one might expect therefore that it should enter the cells still more slowly However, as seen in Fig 1D, the increase in muscle activity developed more quickly in this solution than when urea was used (C) On the other hand, if we compare the responses presented in Fig 1A and D the latter shows a clear-cut delay before the excitation reaches its maximum It thus appears that thiourea immediately after administration exerts an osmotic action on the cells, but the duration of the transient is significantly shorter than with urea

Ethylene glycol has about the same molecular weight as urea, but, in contrast to the latter substance it does not seem to be able to slow down cellular swelling and excitation to any degree The response to a solution in which 20 mmoles NaCl/l had been replaced with ethylene glycol, 40 mmoles/l (Fig 1E) thus did not differ from the response in A where no osmotic substitution was used Another related substance, propylene glycol, of larger molecular weight also failed entirely to prevent or delay the excitatory response (F)

Among other substances used as substitutes for NaCl in this type of experiment erythritol seemed to be as effective as sucrose in preventing the excitatory effect when the exposure periods were short as in Fig 1 After longer periods in the erythritol solution (30 min or more) the muscle showed some increase of activity Substitution of glycerol for NaCl did not prevent excitation due to swelling but the onset of the response was somewhat slower than with urea With solutions containing tetramethylene glycol (1,4-butanediol) or pentamethylene glycol (1,5-pentanediol) the excitatory response reached its maximum as quickly as with the other glycols shown in Fig 1E and F Apart from the changes in contraction fre-

quency, associated with cellular swelling the larger glycols also caused a reduction in the amplitude of the contractions (see further below) The effects obtained with urethane resembled those described for tetra- and pentamethylene glycol

As judged from the results reported above the different test substances can be arranged in the folllowing order with regard to their ability to prevent or retard cellular swelling in solutions with decreased NaCl content sucrose>erythritol> glycerol>urea>thiourea>ethylene glycol = propylene glycol = tetramethylene glycol = pentamethylene glycol = urethane

2 Hyperosmotic solutions

Our previous studies showed that hypertonic solutions, produced by addition of sucrose to the normal medium cause a sustained inhibition of the spontaneous activity in the smooth muscle of the portal vein and that this inhibition is associated with a reduction in cell volume (Johansson and Jonsson 1968 Arvill *et al* 1969) It was also shown in these studies that hyperosmotic solutions containing urea instead of sucrose produced only a transient inhibition followed by a return to control activity (see also Fig 2D below) The response to urea can be explained on the basis of an initial shrinkage of the muscle cells and a recovery to control volume in conjunction with the entrance of urea into the intracellular space Readministration of normal solution then led to a transient excitation due to the osmotic action of the intracellular urea cf Fig 2D below)

Hyperosmotic media produced by adding other non-electrolytes to the standard Krebs solution have been used in the present study Fig 2 shows a series of recordings obtained in one of these experiments For comparison with the effects of other substances the sustained inhibitory response to hyperosmotic sucrose Krebs is shown in Fig 2A The response to a solution containing 100 mmoles erythritol/l is illustrated in Fig 2B Muscle activity was slowed down clearly over the whole period of exposure also with this substance and readministration of normal solution led to a very moderate increase of contraction frequency before control activity was resumed Hyperosmotic solutions produced by adding erythritol to the standard edium thus gave a response pattern which very much resembled that produced by rosmotic sucrose Krebs This would indicate that erythritol can exert a sustained otic action on the smooth muscle cells However with prolonged exposure peri 30 to 60 min the inhibitory response was better sustained in hyperosmotic sucrose than in hyperosmotic erythritol Krebs and with prolonged exposure to erythritol there was also a rather pronounced excitation on return to normal solution In the light of our previous studies these observations suggest that erythritol does enter the smooth muscle cells but that this is a slow process

Addition of glycerol to the standard Krebs solution produced a transient inhibition followed by a return to control activity (Fig 2C) Re administration of normal solution led to a marked but short lasting excitation a phenomenon probably related to a transient swelling of the cells caused by the osmotic action of intracellular glycerol

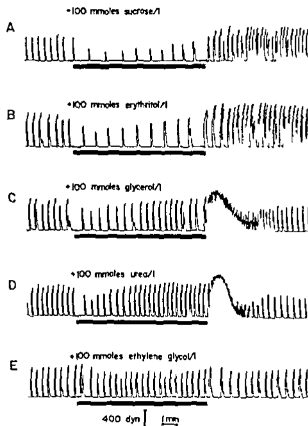


Fig 2 Mechanical responses of a portal vein to hyperosmolarity, produced by adding different non electrolytes to the standard Krebs solution

In principle, the same sequence of events was seen with hyperosmotic urea-Krebs (Fig 2D), but it should be noticed that the duration of the osmotic transients was shorter than with glycerol

Fig 2E illustrates the insignificant effects of hyperosmolarity when produced by addition of ethylene glycol With this substance there was no obvious inhibition during the exposure and no excitatory phase appeared on return to isosmotic Krebs solution This observation is compatible with the conclusion in section 1 above that ethylene glycol penetrates the plasma membranes of the smooth muscle to easily that it fails to exert any obvious osmotic effects on the cells

Similarly, there was no inhibition of contraction frequency during the exposure period nor any excitation on return to normal solution with the larger glycols but as shown in Fig 3 these substances caused a progressive impairment of tension development This latter change of activity which was obtained also with urethane is not typical of the responses to hyperosmolarity *per se*

Hyperosmotic solutions produced by addition of thiourea exerted a very short lasting initial inhibition and there was some excitation on return to normal solution

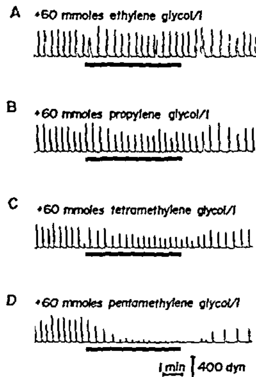


Fig 3 Mechanical responses of a portal vein to hyperosmotic solutions obtained by adding different glycols to the standard Krebs solution

Thiourea like urethane and the large glycols interfered with contraction amplitude when used in high concentrations

The results reported for hyperosmotic solutions in this section suggest the same order of permeability to the different test substances as obtained in section *1*, where the substances were used as osmotic substitutes for NaCl

3 Quantitative aspects of the membrane permeability of vascular smooth muscle cells

The experiments and calculations presented in this section represent an attempt to use the osmotic responses of the portal vein to obtain some approximate quantitative data on the permeability of cell membranes in vascular smooth muscle

The ability of a solute to exert osmotic actions over a membrane can be expressed quantitatively in terms of the Staverman reflexion coefficient σ (Staverman 1951) This coefficient varies from a value of one for ideally non permeant molecules down to (or below) zero for solutes which penetrate as easily as (or easier than) the solvent

When considering the plasma membrane of the vascular smooth muscle cells the results of this and our previous studies suggest that the reflexion coefficient for sucrose is close to or equal to unity The value for ethylene glycol on the other hand should be closer to zero For glycerol urea and thiourea which exert transient osmotic effects σ should lie between these two values The osmotic reflection coefficient is

related to the ratio of the molecular radius and the radius of the pores through which the molecules are assumed to pass the membrane (see below)

A '*The equivalent pore radius*' The pathways for transmembrane diffusion of substances with low lipid solubility are usually looked upon as fluid filled channels which are then often treated schematically as a system of cylindrical pores This model offers a possibility to quantify the size of the channels in terms of an equivalent or effective pore radius One way of estimating the pore radius is to study the permeability of the membrane to several water soluble substances of graded molecular sizes in order to find the largest molecule which can penetrate and the smallest one which cannot A problem is however, that for several reasons, the membrane does not behave in an all-or-none manner in these respects

Observations made in this and in our previous studies (Johansson and Jonsson 1968, Arvill *et al* 1969) indicate that erythritol with a molecular radius of about 3 1 Å enters the vascular smooth muscle cells at a slow rate whereas the sucrose molecule with a radius of 4 4 Å is quite effectively excluded It would seem therefore that the radius of the pores should be somewhere around 4 Å

Estimations of equivalent pore radius may also be made by studying water-soluble substances that penetrate the membrane more easily As mentioned above the osmotic reflexion coefficient is dependent on the ratio of molecular radius and pore radius This ratio can be calculated if σ is known

An attempt has been made in the following experiments to determine the reflexion coefficient for the passage of urea into the smooth muscle cells The method used here for determination of σ is ultimately based on principles worked out by Goldstein and Solomon (1960) in their estimation of the equivalent pore radius in red blood cells Basically, it may be described in the following way Consider first a situation in which some of the non permeant particles of the extracellular fluid are suddenly replaced by the same number of penetrating molecules, for instance urea In this case the cell will start to swell immediately If on the other hand the decrease in the concentration of the non permeant substance is "over compensated" by an excess of the permeant substance in the medium a biphasic response may result with a transient shrinkage preceding the increase in cell volume It should be possible to find a concentration of the permeant substance at which there is no immediate change in cell volume *i e* $dV/dt = 0$ at $t=0$ Goldstein and Solomon (1960) termed this the "isosmotic concentration" and showed that it is related to the osmotic reflexion coefficient of the permeant substance at t_0 according to the equation

$$\sigma_{t_0} = \frac{\sum_j C_j^i - \sum_j C_j^o}{(C_s^o)_{isos}} \quad (1)$$

The nominator on the right side represents the difference between the sum of the concentrations of the intracellular non permeant substances and the sum of the concentrations of the extracellular non permeant substances whereas the denominator

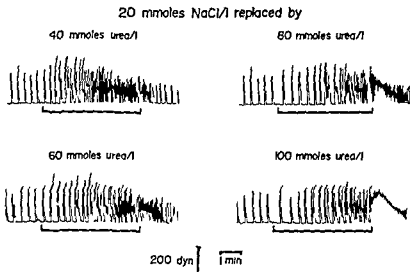


Fig 4 Mechanical responses of a portal vein to solutions in which 20 mmoles NaCl/l were replaced by different amounts of urea

is the isosmotic concentration of the permeant molecule In the practical application the nominator will be represented by the above-mentioned decrease in the osmolar concentration of the non-permeant particles in the medium and the denominator by the isosmolar concentration of the test substance, also expressed in mOsm

Goldstein and Solomon (1960) measured optically the volume changes of red blood cells within fractions of a sec after administration of different test solutions and calculated osmotic reflextion coefficients for several penetrating substances from their isosmotic concentrations With the smooth muscle preparation of the portal vein it takes a certain time for the test substance (urea) to equilibrate with the extracellular space (*cf* Fig 5 below) and the time resolution of methods for determination of changes in cell volume will therefore not in any way be comparable to that in Goldstein's and Solomon's studies on red blood cells In the following experiments an attempt has been made to estimate the direction of the "immediate" change in cell volume by merely recording the mechanical responses of the muscle Fig 4 illustrates one of the experiments In the different test periods the muscle was exposed to solutions containing 102 mmoles NaCl/l *i e* 20 mmoles less than the normal Krebs solution Urea had been added to concentrations of 40, 60, 80 and 100 mmoles/l respectively With 40 mmoles urea/l (Fig 4A) the increase in the frequency of muscle contractions attributed to the swelling of the cells, seemed to occur from the very beginning of the exposure period The intervals between the contractions in the initial phase of the exposure to 60 mmoles urea/l (Fig 4B) were, on the other hand, quite comparable to those of the control situation and excitation was not apparent until after the first min Furthermore at 80 and 100 mmoles

urea/l (C and D) there was a clear cut slowing of muscle activity in the first part of the exposure period indicating that a transient shrinkage of the cells preceded swelling and excitation If 60 mmoles/l (59 4 mOsm) is thus taken as an approximate isosmotic concentration of urea balancing initially the 20 mmoles NaCl (36 6 mOsm) we obtain from equation (1) a value for σ of approximately 0 62 From the practical point of view sodium and chloride ions can be treated as non permeant particles in this calculation Support for this comes from the observation that activity in the portal vein is inhibited to about the same extent when the muscle is exposed to a certain degree of hyperosmolarity produced by sucrose, NaCl and sodium ethanesulphonate, respectively (Johansson unpublished observations)

Fourteen expts of the kind described in Fig 4 have been performed with reductions in NaCl concentration varying between 20 and 30 mmoles/l They indicate that the osmotic reflexion coefficient for urea lies somewhere between 0 6 and 0 7, it is obvious that the value cannot be given with any greater accuracy We will use 0 65 as a reasonable approximation for the following calculations

The osmotic reflexion coefficient is related to the radius of the solute molecule, a the radius of the solvent molecule, a_w, and the radius of the pores r, according to the following formula (see Renkin 1954, Goldstein and Solomon 1960)

$$1 - \sigma = \frac{\left[2\left(1-\frac{a}{r}\right)^2 - \left(1-\frac{a}{r}\right)^4\right]\left[1-2\,10\frac{a}{r}+2\,09\left(\frac{a}{r}\right)^3-0\,95\left(\frac{a}{r}\right)^5\right]}{\left[2\left(1-\frac{a_w}{r}\right)^2 - \left(1-\frac{a_w}{r}\right)^4\right]\left[1-2\,10\frac{a_w}{r}+2\,09\left(\frac{a_w}{r}\right)^3-0\,95\left(\frac{a_w}{r}\right)^5\right]} \quad (2)$$

If we know the molecular radii of urea and water we should be able to use the determination of σ for a calculation of the equivalent pore radius of the smooth muscle cell membranes The values given for the radius of the urea molecule vary, however, depending on how it is determined The uncorrected figure obtained from the Stokes Einstein equation is only 1 6 Å whereas a calculation based on density and molecular weight results in 2 61 Å (Renkin 1954) Measurements on a steric model of the urea molecule gave a mean radius of 2 03 Å (Goldstein and Solomon 1960) Using the latter value the radius of 1 5 Å for the water molecule as given by the same authors, and the value of 0 65 for σ in equation (2) we obtain by successive approximations a pore radius of 3 7 Å corresponding to an a/r ratio of 0 55

A few experiments were also performed to estimate the osmotic reflection coefficients for glycerol and ethylene glycol using the same approach as with urea The isosmotic concentration' of glycerol was lower than that of urea when the two substances were alternatively used as substitutes for NaCl in the same experiments The reflexion coefficient for glycerol seemed to be somewhat above 0 7

The experiments in Fig 1 indicated that ethylene glycol was entirely ineffective as an osmotic substitute for NaCl It was considered of interest to see whether a marked 'over compensation of the reduced NaCl concentration by a large amount of ethylene glycol would be able to delay the excitatory response However, eve

when 300 mmoles ethylene glycol were used to replace 20 mmoles NaCl/l an immediate excitation occurred It thus did not seem possible to obtain an 'isosmotic concentration' of this substance indicating again that its reflexion coefficient is low

B Total pore area and number of pores per cell Data from our previous study of uptake of urea-^{14}C in the isolated portal vein (Arvill *et al* 1969) can be combined with the present results concerning the permeability to urea to reveal some further quantitative aspects of the porous pathways of the membrane The ratio between the radius of the urea molecule and the radius of the pores, a/r, determined from the osmotic reflexion coefficient above enables us to calculate the restricted diffusion coefficient D' for the passage of urea through the cell membranes of the vascular smooth muscle D' is related to the free diffusion coefficient, D, and to a/r according to the equation (see *e g* Landis and Pappenheimer 1963)

$$\frac{D'}{D} \approx \left(1-\frac{a}{r}\right)^2 \left\{1 - 2\ 10\frac{a}{r} + 2\ 09\left(\frac{a}{r}\right)^3 - 0\ 95\left(\frac{a}{r}\right)^5\right\} \tag{3}$$

At 37° C the free diffusion coefficient for urea is $1\ 5 \times 10^{-5}$ cm²/sec This and the a/r ratio of 0 55 obtained above give a value for D' of $3\ 9 \times 10^{-7}$ cm²/sec

If we assume that the cell membrane is the only important barrier for the diffusion of urea in the smooth muscle and if we make certain assumptions as to the geometry of this membrane it should now be possible to apply Fick's law of diffusion to our previous data on the uptake of urea into the portal vein (Arvill *et al* 1969) The values obtained in that study for the distribution volumes of urea ^{14}C after variable periods of incubation have been plotted semilogarithmically in Fig 5 To facilitate the analysis of the uptake data the ordinate in Fig 5 has been made to represent the difference between the maximal distribution volume, obtained after 60 min of incubation and the distribution volumes at shorter incubation periods After an initial rapid phase which most probably represents diffusion into the extracellular

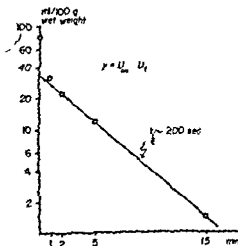


Fig 5 Semilogarithmic representation of distribution volumes of urea ^{14}C in portal vein after different periods of incubation Original data reported by Arvill et al (1969) The ordinate values represent the difference between the distribution volume obtained at full equilibration and that obtained with shorter incubation periods

space there is a slower component with a $t_{\frac{1}{2}}$ of about 200 sec representing cellular uptake. *It is worth noticing that the intercept of the slow component and the ordinate corresponds to a value of 33 ml/100 g wet weight which agrees with the value of 34 ml/100 g for the amount of intracellular water obtained by subtracting sucrose* space from total water (Arvill *et al.* 1969).

According to Fick's law of diffusion the intracellular concentration of urea ^{14}C should increase in these experiments at a rate given by

$$C_i = C_0\left(1 - e^{-\frac{D\, f\, A}{X\, V}\, t}\right) \tag{4}$$

In this equation C_i is the intracellular concentration at time t, C_0 is the extracellular concentration (being constant), X is the thickness of the cell membrane and A/V is the surface to volume ratio of the cell. The total pore area over which diffusion of urea into the cell takes place is f · A and a calculation of f thus gives us the fraction of the cell membrane which is taken up by the pores. Assuming that the vascular smooth muscle cell is a cylinder with a radius of 2 μ and a length of 50 μ and assuming a membrane thickness of 80 Å (Rhodin 1962) we obtain

$$f = \frac{\ln 2 \cdot X \cdot V}{t_{\frac{1}{2}} \cdot D \cdot A} = 7 \cdot 10^{-7}$$

The total pore area per cell, f · A, would then be approximately 4.4×10^{-12} cm² and since each pore has an area of $\pi \cdot (3.7 \times 10^{-8})^2$ cm² there would be only about 10^3 pores per cell. The number of pores per cm² would be 1.6×10^8.

Discussion

Many different types of living cells behave like osmometers in that they adjust their volume in response to variations in extracellular tonicity so that osmotic equilibrium over the cell membrane is restored. This is true at least with moderate changes in osmolarity. As far as muscle cells are concerned such osmotic adjustments of cell volume have been described for instance in amphibian skeletal muscle (Blinks 1965), in heart muscle (Page and Storm 1966), in the intestinal smooth muscle of guinea pig taenia coli (Brading and Setekleiv 1968) and in the vascular smooth muscle of rat portal vein (Arvill *et al.* 1969, Jonsson 1969). The regular spontaneous activity of the portal vein, which is maintained for hours in normal Krebs solution, and the consistent responses of this muscle to variations in the tonicity of the extracellular fluid seemed to offer a possibility for studying the osmotic actions of different substances simply by recording the contractile responses.

Osmotic effects on living cells of solutions containing a large variety of organic and inorganic substances have previously been subjected to extensive investigations and such studies have helped to form the current views on the permeability characteristics of cell membranes. Overton's classical studies on plant cells and later on amphibian skeletal muscle cells led to his important concept of the role of lipid solubility for the rate of penetration of substances into cells (see Overton 1

However later experiments particularly with certain types of plant cells (*e g* Ruhland and Hoffman 1925 Barlund 1929, Collander and Barlund 1933, Collander 1954) showed that the passage of substances through the cell membranes could be greatly dependent also on the molecular size and thus suggested a kind of sieve-effect of the membrane From these and other investigations it appears that the ability of a substance to penetrate into the cells is determined both by its lipid solubility and by its molecular size, suggesting the existence of two alternative pathways for passive transmembrane diffusion *i e* lipid regions and water filled pores The latter pathways should be important for the passage of water and of small molecules with low lipid solubility

The different substances examined with regard to their osmotic action on the smooth muscle of the portal vein have been arranged in Table I according to their relative rates of penetration as indicated by the findings in section 1 and 2 above It is evident from other data given in Table I that this order of permeability cannot be simply related to molecular size nor can it be wholy accounted for on the basis of lipid solubility when this is expressed in terms of oil/water partition coefficients The apparent rates of entrance of sucrose erythritol glycerol and urea are compatible with the concept of porous pathways for these substances The differences between urea and ethylene glycol which are of comparable molecular size are so conspicuous however that one has to assume qualitative differences in their mechanisms of penetration It seems unlikely that the slightly larger lipid solubility of ethylene glycol should account for the difference This latter possibility seems even more remote if we consider also the results with thiourea which has a still higher oil/water partition coefficient but apparently a slower rate of penetration than ethylene glycol

Previous studies of other animal cells are of interest in connection with the present findings regarding the permeability of vascular smooth muscle to glycerol urea ethylene glycol and thiourea In amphibian sartorius muscle Overton (1902) found transient changes in muscle weight with solutions containing ethylene glycol but the rate of penetration of this substance was faster than that of glycerol which in turn was found to penetrate more rapidly than urea Erythrocytes from several mammalian species showed a significantly higher permeability to urea than to ethylene glycol whereas the situation was the opposite in avian erythrocytes (Hober and Ørskov 1933 In these respects glycerol resembled ethylene glycol rather than urea Hober and Ørskov also found a much lower permeability to thiourea than to urea in mammalian red blood cells whereas the difference was small or even reversed in the erythrocytes of birds

Returning to the smooth muscle of the portal vein it thus appears from the comparison with urea that some facilitating mechanism other than mere lipid solubility would be necessary to account for the passage of ethylene glycol and perhaps also for thiourea One possibility would be that the lipoid material of the membrane has a much higher affinity to these substances than indicated by their oil/water partition coefficients (*cf* Collander 1947 The apparent rapid penetration of the larger glycols and of urethane into portal vein is in accordance with Overton s rule about

the importance of lipid solubility These substances in higher concentrations cause deterioration of muscle activity (Fig 3C and D), a phenomenon which may be related to the general "narcotic" action of alcohols urethanes, ethers *etc* on cell membranes (Davson 1964 p 322)

The quantitative calculations concerning the porous component of the cell membranes performed in section *3* above must obviously be judged with caution due to the limitations of the experimental approach and to the question of the applicability of the diffusion equations to pores of molecular size But such reservations apply more or less to all attempts to obtain a model of the permeability of biological membranes The porous pathways may entirely be a product of our imagination (or lack of imagination) and what appears to be a molecular sieve effect might be explainable on the basis on other concepts

The estimation of the equivalent pore radius is an important step in the calculations presented in section *3* Various procedures have been used previously to obtain evidence for the existence of porous pathways in cell membranes and to determine the size of these pores (see Solomon 1968) In a study of human red blood cells Paganelli and Solomon (1957) applied Poiseuille s law to measurements of the osmotic flux of water over the cell membranes and Fick s law of diffusion to the uptake of tritiated water By combining these equations the authors arrived at an equivalent pore radius of 3 5 Å Goldstein and Solomon (1960) obtained a value of 4 2 Å for human erythrocytes from their determinations of osmotic reflexion coefficients for several different non electrolytes These approaches were both used in a recent study of dog erythrocytes which, in comparison with human red cells, are characterized by a higher permeability to water and water soluble substances (Rich *et al* 1967) The two methods indicated an equivalent pore radius in dog erythrocytes of 5 9 and 6 2 Å respectively

The method used in the present study is based on the principles developed by Goldstein and Solomon (1960) in their study on human red blood cells but the present experimental approach is certainly more indirect and not as accurate as theirs The results reported in section *3* above indicated a reflexion coefficient for urea between 0 6 and 0 7 which corresponds to an equivalent pore radius between 3 5 and 4 0 Å A radius of 3 7 Å was obtained with the value for σ of 0 65 chosen for the calculations in section *3* When considering the molecular radii of urea glycerol erythritol and sucrose (Table 1) their relative abilities to penetrate the cell membranes are compatible with this size of pores

It thus appears as if the pores in the vascular smooth muscle cells are quite comparable in size to those of human erythrocytes Calculations of total pore area however result in very different values for the two types of cells which is not very surprising perhaps in view of their different functional characteristics Paganelli and Solomon (1957) concluded from their studies of the water permeability of erythrocytes that a fraction of approximately 10^{-4} of the total surface area of the cell is occupied by the pores The present calculations based on the uptake of urea ^{14}C into the smooth muscle cells indicated that the channels available for diffusion of

this substance corresponded to a much smaller fraction, 7×10^{-7}, of the cell membrane In the membrane of the vascular smooth muscle cells there would then be about 1.6×10^{8} pores/cm² compared to about 2.3×10^{11} in the membrane of human erythrocytes The number of about 10^{3} pores per cell in the smooth muscle of the portal vein seems rather small indeed It appears that this small population of pores would not allow a very rapid transfer of water in response to variations in osmolarity We may have to assume that water can penetrate the cell membranes also by other pathways than those available to urea but this question cannot be settled until the water permeability has been studied directly either by means of tritiated water or by detailed analysis of the time course of the osmotic changes in cell volume

Further studies of the osmotic behaviour of the portal vein and of its permeability to different substances may help to elucidate in functional terms the physical and chemical characteristics of the cell membranes in this type of vascular smooth muscle and may contribute to the understanding of the control of its contractile machinery

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Effects of Some Metabolic Co-Factors and Inhibitors on Transmitter Release and Uptake in Isolated Adrenergic Nerve Granules

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Abstract

Euler, U S v and F Lishajko *Effects of some metabolic co factors and inhibitors on transmitter release and uptake in isolated adrenergic nerve granules* Acta physiol scand 1969 77 298—307

Various nucleotides and metabolic inhibitors have been tested on the release reuptake and net uptake of noradrenaline (NA) in isolated splenic nerve granules Cytidine, inosine and uridine triphosphate facilitated uptake of NA to a similar degree as adenosine diphosphate but less than adenosine triphosphate Cyclic AMP had no effect

Various inhibitors of the respiratory chain and oxidative phosphorylation (rotenone chlorpromazine antimycin and oligomycin) inhibited both release and uptake of NA at 10 [illegible]—10^{-4} M Atractylate had no action at 10^{-3} M Cyanide and azide at 10^{-3} M had no or a slight action only

The uncouplers dinitrophenol carbonyl cyanide *m* chlorophenyl hydrazone desaspidin and pentachlorphenol all increased the release rate of NA from granules and inhibited reuptake

The results suggest that the ATP facilitated uptake process requires electron transport and that the ability of the granules to retain bound NA is dependent on an energized state of the system At least certain steps of electron transport-coupled phosphorylation occurring in mitochondria appear to be involved in amine release and uptake in granules

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On incubation of transmitter granules from bovine splenic nerve homogenates the release rate of noradrenaline (NA) is mainly dependent on the temperature pH and NA concentration of the incubation medium (Euler and Lishajko 1963 a 1967) In a NA free medium the release constant is 1 8 × 10 [illegible] or 1 8 per cent per minute at 20° and pH 7 5 The net release rate approaches zero at a NA concentration in the medium of 10^{-4} M as a result of concomitant reuptake (Euler Stjärne and Lishajko 1963)

In granules which have been partially depleted by previous incubation a net uptake of NA occurs in the presence of the amine in the medium This uptake is small at 10 [illegible] M NA but is greatly enhanced by addition of adenosine triphosphate (ATP) and Mg^{++} (1 3 mM) to the medium (Euler and Lishajko 1963 b)

The presence of ATP in nerve granules (Schümann 1958) and the facilitation of uptake of transmitter after addition of ATP to the medium raises the question whether endogenous ATP is also involved in the "spontaneous" reuptake of NA during incubation of granules, and whether the mechanism by which ATP might act is through the mechanism of electron transport linked phosphorylation

In order to obtain more information about the nature of the processes governing release and uptake of the transmitter in the storage granules some experiments have been carried out in which these events have been studied in the presence of various nucleotides and a number of metabolic inhibitors

Methods

All experiments were made with bovine splenic nerve granules obtained by squeezing nerves between rollers or by homogenization of the desheathed nerves in an Ultra Turrax apparatus (Janke & Kunkel, Freiburg) with 0 13 M potassium phosphate pH 7 5, about 10 ml per g nerve tissue at ice water temperature

The nerve homogenates or press juice was centrifuged for 10 min at $9000 \times g$ in order to remove larger particles and the supernatant used for the incubation experiments Under these conditions the NA concentration in the medium attained a value of $1—3 \times 10^{-6}$ M Each tube contained 8 ml particle suspension corresponding to about 0 5—1 g nerve per tube

[illegible] were incubated for 60 min at 20° C and [illegible] at $50\,000 \times g$ after the incubation In all [illegible] um the sediment was washed with fresh [illegible] extracted with 0 5 ml 0 4 M perchloric acid and an aliquot after addition of 2 5 ml water and centrifugation used for fluorimetric estimation of NA

In the net uptake experiments the NA content of the granule suspension was depleted by 60—70 per cent by incubation for 10 min at 37° The suspension was then further incubated for 30 min at 20° during which time net uptake occurred At the end of the incubation period the NA content in the granules was measured as described above

In some experiments the reuptake and net uptake has been studied in the presence of radioactive dl NA either as ^{3}H-dl NA or ^{14}C dl NA The incorporation of labelled NA is expressed as the ratio of the specific activities of NA in the sediment and in the supernatant (SA_{sed}/SA_{sup}) The radioactivity was measured in a Packard liquid scintillation spectrometer as described previously (Euler and Lishajko 1967)

Results

Effect of nucleotides on release reuptake and net uptake of NA in nerve granules
Adenine nucleotides

As described in previous papers (Euler and Lishajko 1963 b 1967) ATP strongly retards the net release of NA from incubated nerve granules in the presence of Mg^{2+} This effect is due in part to increased reuptake of NA as evidenced by the incorporation of radioactive NA It has been observed however that the labelled

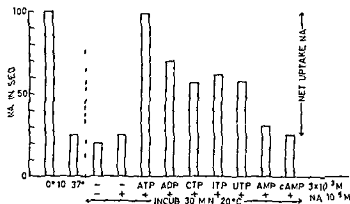


Fig 1 Splenic nerve granules incubated in 0.13 M K phosphate pH 7.5 After preincubation 10 min 37°, continued incubation 30 min 20° with addition of nucleotides and noradrenaline as indicated Ordinate per cent NA remaining in granules after incubation (Net uptake experiment)

portion of the reuptake in the presence of ATP is smaller than what may be expected if the release was unchanged This suggests either some inhibition of the release, or reuptake of unlabelled NA, possibly as a result of incomplete mixing of newly released NA with the medium

ADP enhances reuptake and net uptake, although markedly less than ATP The degree of enhancement shows variations from one experiment to another in comparison with ATP On an average, the effect of ADP on reuptake and net uptake is about one half of that of ATP AMP exerts only a weak action on NA release or on NA net uptake in concentration 1—3 mM No definite effect was observed with cAMP (Fig 1)

Effect of CTP, ITP and UTP

These three nucleotides stimulate reuptake and enhance the net uptake of NA in nerve granules to about the same extent in a concentration of 3×10^{-3} M ITP exerts a slightly stronger action both on reuptake and net uptake Quantitatively the effect is of a similar order as that of ADP (Fig 1)

1 *Inhibitors of the respiratory chain and oxidative phosphorylation*

Rotenone which was tested as inhibitor of NADH oxidation inhibited both release and reuptake in concentrations from 10^{-5} M

Antimycin A an inhibitor of both succinate and NADH oxidation almost completely prevented the reuptake of NA at 10^{-4} M and higher concentrations while moderately inhibiting the NA release (Fig 2) Similarly it inhibited or prevented the ATP facilitated net uptake of NA after previous partial depletion of the granules

Cyanide azide KCN 10^{-3} M inhibited the release of NA by 17—25 per cent but had no effect on the reuptake or net uptake of NA even at 1×10^{-3} M concentration

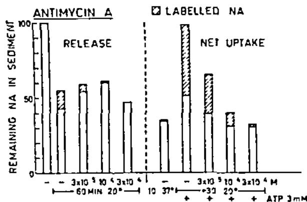


Fig 2 Splenic nerve granules incubated in 0 13 M K phosphate pH 7 5
Left Release expt Ordinate per cent remaining NA in granules after incubation 60 min 20° with antimycin A in different concentrations
Right Net uptake expt as in Fig 1 ATP facilitated NA uptake gradually diminished by addition of antimycin

The effect of NaN_3 differed from that of cyanide in that it inhibited NA uptake and increased the NA release rate at 10^{-} M

Chlorpromazine was tested as an inhibitor of phosphorylation reactions associated with electron transport (Löw 1959) It has previously been shown to inhibit NA release as well as NA uptake in nerve granules in concentrations from 3×10^{-5} M (Euler and Lishajko 1968 a) (Fig 3) This effect it has in common with other adrenergic alfa blockers As seen in Fig 3 NA release is inhibited at 3×10^{-6} M and incorporation of NA blocked at 10^{-4} M, both in release and uptake experiments

The releasing effect at 3×10^{-4} M suggests an action different from that at lower concentrations Similar diphasic effects have been observed for prenylamine and some other drugs (Euler and Lishajko 1968 b)

Atractylate which inhibits transport of ATP and ADP through the mitochondrial barrier, but has no effect on oxidative phosphorylation as such (Heldt Jacobs and Klingenberg 1965) had no effect on release or uptake of NA when given in concentrations up to 10^{-3} M

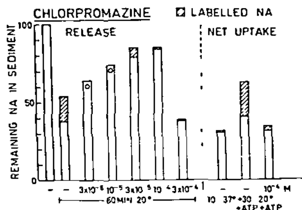


Fig 3 Splenic nerve granules incubated in 0 13 M K phosphate pH 7 5
Left release expt Ordinate per cent remaining NA in granules after incubation 60 min 20° with chlorpromazine in different concentrations (Circle in column = labelled NA not added)
Right Net uptake expt as in Fig 1 ATP facilitated NA uptake prevented by chlorpromazine 10^{-4} M

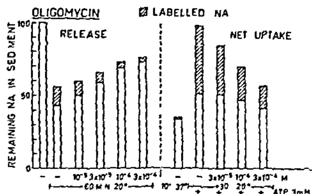


Fig 4 As in Fig 3 but with addition of oligomycin Note inhibition of release diminished reuptake and inhibition of ATP facilitated net uptake

Oligomycin This compound, which inhibits ATP ase and oxidation coupled to phosphorylation, exerted a marked inhibition of NA release, about 60 per cent at 3×10^{-4} M It also inhibited the ATP facilitated net uptake of NA in partially *depleted granules (Fig 4) It differed in action from antimycin in being some 10* times less active on the net uptake of NA and also in lacking the increased exchange effect on NA between medium and granules

2 *Other enzyme inhibitors*

Arsenate in concentrations up to 1.8×10^{-2} M had no definite effect on release or reuptake of NA

NaF No effect on release or uptake was observed with NaF in a concentration of 10^{-2} M

3 *SH-reagents*

Iodoacetate 10^{-3} M moderately or slightly inhibited the release rate Addition of KCN and iodoacetate 10^{-3} M together caused a slightly greater inhibition than each of the two compounds separately In 3×10^{-4} M concentration neither iodoacetate nor KCN altered the NA release rate when given separately whereas the combined effect was 35 per cent inhibition

p chloro and p hydroxy mercuribenzoate o iodosobenzoate and *N ethyl maleimide* (NEM had no significant effect on the NA release rate at 3×10^{-4} M or *caused a slight inhibition In 10^{-3} M concentration the p mercuri benzoates caused* a rapid and almost complete NA release which also occurred at +2 *o* iodosobenzoate and NEM had no definite effect on the release at 3×10^{-4} M but NEM inhibited net uptake by 40 per cent

4 *Uncouplers*

nitrophenol DNP

NP increases the NA release in concentrations from 10^{-4} M upwards Above 3×10^{-4} M the release is increased over and above that observed in the presence of ferricyanide basic release rate indicating that the effect is not due merely to

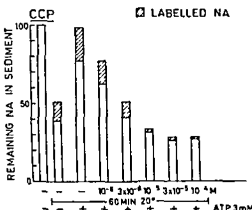


Fig 5

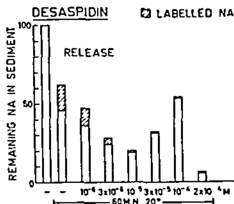


Fig 6

Fig 5 Splenic nerve granules incubated 60 min 20° in 0.13 M K phosphate at pH 7.5. Release expt with addition of ATP and CCP. ATP induced inhibition of release and increased reuptake prevented by CCP 3×10^{-6} M. Increased NA release and reduced reuptake at higher concentrations of CCP.

Fig 6 Splenic nerve granules incubated 60 min 20° in 0.13 M K phosphate at pH 7.5. Release expt. Triphasic action of desaspidin on NA release from granules and inhibition of reuptake of NA from 3×10^{-6} M.

inhibition of reuptake. This may, however, be a contributing factor at concentrations from 10^{-4} M on, since the proportion of incorporated radioactive NA begins to decrease at this level.

The net uptake of NA in the presence of ATP is markedly reduced by DNP at 3×10^{-4} M. The net uptake of NA decreases rapidly at higher concentrations and is small at 3×10^{-3} M.

Dinitrophenylhydrazine (DNPH)

The hydrazine derivative of DNP acts in principle like DNP, increasing the release rate of NA from 3×10^{-5} M on. It inhibits reuptake and net uptake of NA in about the same way as DNP. Thus at 6×10^{-4} M the incorporation of ^{3}H NA is less than 10 per cent of that in the control.

Carbonyl cyanide m chlorophenylhydrazone (CCP)

Heytler and Prichard (1962) have described a new class of uncoupling agents, the carbonyl cyanide phenylhydrazones. In the present experiments we have used the *m* chloro derivative of CCP (kindly given to us by professor A. Revnard, Buffalo, N.Y.).

CCP increases the release rate of NA in concentrations from about 10^{-6} M on. Part of this effect is apparent and is due to inhibition of reuptake, but part is due to a direct releasing effect as indicated by the fact that release exceeds that caused by ferricyanide. In addition to the direct release effect the incorporation of ^{14}C NA is inhibited by about 85 per cent at a CCP concentra

TABLE I Effect of various metabolic and enzymic inhibitors on release reuptake and ATP facilitated net uptake of NA in isolated nerve granules (decrease increase or no change)

	Release	Reuptake	Net uptake	Conc M
Chlorpromazine	— —	— —	— —	10^{-4}
Rotenone	— —	— —	— —	10^{-4}
Antimycin A	—	— — —	— —	10^{-4}
Oligomycin	— —	— —	— —	3×10^{-4}
Atractylate	0	0	0	10^{-3}
KCN	(—)	0	0	$1—4 \times 10^{-3}$
NaN_3	0	0	0	10^{-3}
p CMB p HMB	0	0	0	10^{-4}
o iodosobenzoate	(—)	0		3×10^{-4}
NEM	(—)	0	— —	3×10^{-4}
NaF	0	0	0	10^{-3}
Iodoacetate	(—)	(—)		10^{-3}
K arsenate	0	0		2×10^{-3}
DNP	+ +	— —	— —	10^{-3}
DNPH	(+)	— — —	— — —	6×10^{-4}
CCP	+ +	— —	— —	10^{-5}
Desaspidin	+ +	— —		3×10^{-4}
Pentachlorphenol	+ +	— —		10^{-4}

The ATP facilitated reuptake of NA in release experiments is annulled by CCP in concentrations of 3×10^{-6} M (Fig 5)

The net uptake of NA in partially depleted granules is decreased by CCP in concentrations of 10^{-6} M and higher as shown by the reduced incorporation of ^3H NA

As regards its direct releasing effect and the inhibition of NA uptake CCP is similar in action to DNP although it appears to be some 100 times more effective

Desaspidin

Desaspidin acts as a potent uncoupler of oxidative phosphorylation in rat liver mitochondria (Runeberg 1962) It also stimulates the latent ATPase in low concentrations but has an inhibitory action in higher concentrations Its biphasic action is more marked than that of DNP (Runeberg 1963)

As seen in Fig 6 desaspidin (kindly placed at our disposal by dr L Runeberg) increases the release rate from about 10^{-6} M on and at the same time inhibits the reuptake of NA The release rate increases to a maximum at 10^{-5} M whereafter it decreases up to 10^{-4} M At 2×10^{-4} M the release rate is again strongly increased Reuptake is almost completely inhibited from 10^{-5} M on and is thus observed during all of the three phases In a concentration of 10^{-5} M desaspidin caused no effect on the release at 2 C whereas it strongly increased the release of NA at 20°, indicating a specific effect

Pentachlorphenol

Like the other uncouplers tested, pentachlorphenol increased the NA release from 3×10^{-5} M on and at the same time inhibited the incorporation of labelled NA by reuptake The strong release at 3×10^{-4} M still partly persisted at 2° C, suggesting an unspecific action

Discussion

The triphosphates of the ribosides cytidine, inosine and uridine markedly enhanced the NA uptake in granules (Fig 1) Their effect was of the same order as that of ADP On the other hand AMP had only a weak facilitating effect on the NA uptake and with cAMP no effect could be observed This is in harmony with the concept that cAMP mediates catecholamine action at the effector side *i e* subsequently to the interaction at the receptor site (Sutherland Robison and Butcher 1968)

The high temperature dependence of the release of NA on incubation of nerve granules in various media and its character of a first order reaction suggests that the release process is in some way metabolically regulated Preliminary observations (Giacobini unpubl) indicate that the oxygen consumption of isolated granules amounts to only a few per cent of that in mitochondria On the other hand the release and uptake of NA in a suspension of nerve granules is of the order of 10 nmoles per incubation unit in our experiments If these processes are driven by energy producing reactions these would be expected to be of a small order On the other hand it can not be excluded that mitochondria participate in an indirect way to the granule mediated processes *in vivo* possibly by producing ATP

Carlsson Hillarp and Waldeck (1963) studied the action of a number of enzyme inhibitors on the facilitating effect of ATP Mg on the amine uptake in adrenal medullary granules Of the compounds studied the SH reagents appeared to exert the most powerful inhibitory action

Metabolic processes have been implicated in the axonal uptake of amines insofar as this process can be separated from the granular uptake Thus Kirpekar and Wakade (1968) reported that while iodoacetic acid or DNP 5×10^{-4} M inhibited the uptake of amines in the perfused spleen only moderately they had a considerable inhibitory effect when given together

Kirshner and Smith (1966) observed that cyanide and iodoacetic acid each had only little effect but when given together prevented catecholamine secretion by acetylcholine in the perfused adrenal gland Secretion was not inhibited by antimycin or oligomycin given alone but either of them in combination with iodoacetate blocked secretion

Of the inhibitors acting at different sites of the respiratory chain chlorpromazine rotenone and antimycin A inhibited or prevented the release as well as the uptake of NA in granules These compounds completely prevented the ATP facilitated net uptake of NA in partially depleted granules in concentrations of 3×10^{-4} M Cyanide and azide on the other hand had only moderate effects at 10^{-3} M and were not parallel in action

The sulfhydryl reagents *p* chloro and *p* hydroximercuri benzoate *o* iodoso benzoate and NEM had only weak inhibitory actions on the transmitter release The net uptake was inhibited some 40 per cent by NEM in a concentration of 3×10^{-4} M

The rapid breakdown of the release mechanism observed with the *p* mercuri benzoates at 10^{-3} M even at 2° seems to indicate that intact SH groups are necessary for the graded NA release

The observed action of oligomycin as well as of chlorpromazine in low concentrations may be associated with their inhibitory action on ATP formation as found in mitochondria (Lardy Johnson and McMurray 1958) On the other hand atractylate had no action in concentrations up to 10^{-3} M which may be due to the fact that it does not inhibit oxydative phosphorylation as such (Heldt *et al* 1965)

All of the uncouplers tested DNP DNPH CCP desaspidin and pentachlorphenol increased the release of NA from granules and inhibited reuptake and the ATP dependent net uptake thereby differing from the inhibitors rotenone chlorpromazine antimycin and oligomycin which inhibited both uptake and release of NA *The results are suggestive of an ATP-dependent process involved in the uptake* and binding of NA in the granules (*cf* Belleau 1966) A participation of endogenous ATP in the release of NA may also conceivably occur

Although previous and present results strongly suggest that the ATP facilitated net uptake of NA in nerve granules is dependent on metabolic processes the available data do not allow more precise characterizations of the processes involved but they speak in favour of the assumption that at least certain steps of electron transport coupled phosphorylation similar to those involved in mitochondria participate The presence of ATP in the granules and its marked effect on the incorporation of NA from the medium into the granules suggest however the importance of phosphorylating processes presumably coupled with oxidations In this direction speaks also the effect of oligomycin which is considered as a fairly specific agent for the inhibition of oxidative phosphorylation The effect of oligomycin is to *block release as well as uptake of NA in the granules an effect which is on the face* similar to that of reserpine LSD and some other drugs with psychotropic actions Antimycin differs in some respects in its action from oligomycin although it is highly active as an inhibitor of the ATP facilitated uptake of NA These data would be *compatible with the view that NA uptake and binding is dependent on phosphorylation* Exogenous ATP which normally greatly facilitates uptake of NA in the granules is devoid of action in the presence of antimycin which indicates that electron transport is required for the action of ATP

It has also been noticed that in the presence of exogenous ATP and NA the inhibitory effect of this group of compounds is reduced or requires a higher concentration

The uncouplers on the other hand appear to cause an alteration of the conditions leading to reduced ability or inability of the granules to retain the bound NA a *function which therefore might be dependent on an energized state of the system*

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Effect of Acid in the Duodenal Bulb on Gastric Secretory Responses to Sham Feeding[1]

By

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Abstract

NILSSON G *Effect of acid in the duodenal bulb on gastric secretory responses to sham feeding* Acta physiol scand 1969 77 308—315

Gastric secretion was stimulated by sham feeding in Pavlov pouch dogs with esophageal fistulae and isolated pouches of the duodenal bulb Reduction of the intrabulbar pH to pH 3—4 inhibited the acid response to sham feeding Bulbar acidification was more effective in inhibiting acid responses to 1 and 10 min of sham feeding than responses to 30 min of sham feeding Short periods of bulbar acidification were sufficient to activate the bulbar inhibitory mechanism

Perfusion of isolated pouches of the duodenal bulb with 0 1 N HCl inhibits gastric acid secretion in response to a test meal (Andersson and Uvnas 1961), exogenous gastrin (Andersson Nilsson and Uvnas 1965 1967, Andersson and Nilsson, in press) and insulin hypoglycemia (Nilsson 1969)

The present series of experiments was undertaken to study the effect of bulbar acidification on acid responses to vagal activation by sham feeding, which is considered to constitute a more physiological stimulus of gastric acid secretion than insulin hypoglycemia The experiments were performed on Pavlov pouch dogs provided with esophageal fistulae and innervated pouches of the duodenal bulb

Methods

Surgical procedures

7 mongrel dogs weighing 13—18 kg were provided with esophageal fistulae according to a technique elaborated at this laboratory (Olbe 1959) The dogs were also provided with mucosal septal pouches of the stomach Pavlov type) and innervated pouches of the duodenal bulb (Andersson and Uvnas 1961) After each surgical procedure and before the experiments were started the dogs were allowed a period of 2—3 weeks for recovery

Experimental procedures

experiments were started in the morning after the dogs had been fasted for 18—21 hrs l acid output was recorded for 1 hr Gastric secretory responses were collected in 15 min tions and followed for 3—4 hrs The volume was measured and the acidity determined by

[1] A preliminary report of this investigation was presented at the XII th Scandinavian Congress of Physiology Turku, 1966 *Acta physiol scand* 1966 *68* suppl 277

TABLE I Secretory responses to 10 min of sham feeding in Pavlov pouch dogs with and without perfusion of the duodenal bulb with 0 9 % NaCl or 0 1 N HCl

Experimental procedure	Number of expts	Secretion in meq acid (mean and range)				
		Control period (1 hr)	Response to 10 min of sham feeding			
			1st hr	2nd hr	3rd hr	4th hr
Dog 19						
Controls	3	0 02 (0—0 05)	4 36 (4 19—4 64)	3 74 (2 46—5 15)	3 36 (2 10—5 84)	3 11 (1 87—4 14)
0 9 % NaCl in the duodenal bulb for 30 min before and 30 min after the start of the sham feeding procedure	3	0 (—)	4 60 (4 06—5 29)	3 81 (3 51—4 19)	3 73 (3 06—4 68)	3 08 (2 58—3 41)
As percentage of means for controls		—	106	102	111	99
0 1 N HCl in the duodenal bulb for 30 min before and 30 min after the start of the sham feeding procedure	3	0 (—)	0 50 (0 23—0 74)	1 14 (0 87—1 55)	1 85 (1 78—1 90)	1 79 (1 10—2 74)
As percentage of means for controls		—	11	30	55	58
Dog 20						
Controls	3	0 (—)	3 66 (3 06—4 28)	3 36 (2 47—4 18)	2 16 (0 48—3 26)	2 08 (1 29—2 77)
0 9 % NaCl in the duodenal bulb for 30 min before and 30 min after the start of the sham feeding procedure	3	0 (—)	3 73 (2 43—4 67)	3 68 (2 43—4 65)	2 59 (1 10—3 37)	1 35 (0 15—2 05)
As percentage of means for controls		—	102	110	120	65
0 1 N HCl in the duodenal bulb for 30 min before and 30 min after the start of the sham feeding procedure	3	0 (—)	0 (0—0 01)	0 01 (0—0 01)	0 21 (0 05—0 29)	0 49 (0 20—1 00)
As percentage of means for controls		—	0	0	10	24

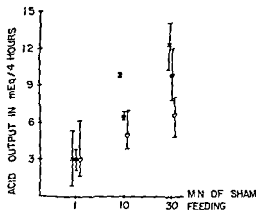


Fig 1 Acid responses to 1, 10 and 30 min of sham feeding (SF) in 3 Pavlov pouch dogs (7 = •, 21 = o, 22 = x) Each symbol represents the mean of 3 expts Vertical bars show the range

control experiments were performed to determine if the acid response to sham feeding was influenced by bulbar perfusion with a solution of an inert agent In these experiments acid secretion was elicited by 10 min of sham feeding The bulbar pouches were perfused with ... the perfusate ... ntrol experi ... uches in the ... in procedure

Three series of experiments were performed In series A the pH in the duodenal bulb was kept constant (pH 1 1—1 4) during 4 hrs by bulbar perfusion with 0 1 N HCl After 1/2 hr of bulbar perfusion ... 30 min ... tead, the du ... pouches we ... with or 1 1 ... 270 min we ... I of the effluent perfusate varied in all experiments between pH 1 1 ...

In series C the effect of different pH-levels in the duodenal bulb on the acid responses to 10 min of sham feeding was studied In each experiment the bulbar pouch was perfused with one buffer solution The perfusions were performed as in series A The degree of secretory inhibition was calculated from the acid output during 4 hrs and expressed as a percentage of the mean acid output during perfusion with buffer solution of pH 7 Bulbar perfusion with this buffer solution does not influence the acid response to sham feeding Sorensen's citrate HCl buffers were used

All bulbar perfusions were performed at a rate of 60 ml per hr The perfusions did not visibly affect the appetite of the dogs The pH of the effluent perfusate collected during 1 or 15 min was determined The technique of bulbar perfusion was described in detail in a previous paper (Andersson Nilsson and Uvnäs 1967)

Results

... is seen in Fig 1 the gastric acid output was dependent on the duration of the ... sham feeding

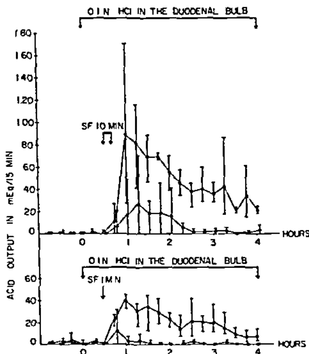


Fig 2 Acid responses to 1 (lower graph) and 10 (upper graph) min of sham feeding (SF) in a Pavlov pouch dog (7) with (closed symbols) and without (open symbols) 0 1 N HCl in the duodenal bulb Each symbol represents the mean of 3 expts Vertical bars show the range

Series A

These experiments were performed on 2 dogs Fig 2 and 3 illustrate the results from each dog Bulbar perfusion with 0 1 N HCl almost completely inhibited the gastric acid responses to 1 min of sham feeding The acid responses to 10 min of sham feeding were less influenced and the acid responses to 30 min of sham feeding still less susceptible to bulbar inhibition

Series B

The results from this series of experiments are presented in Table II and Fig 4 In this series the sham feeding period was constant (10 min) whereas the duration of

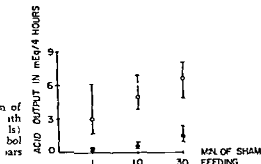


Fig 3 Acid responses to 1, 10 and 30 min of ... ith ... ls) ... bol ... bars show the range.

TABLE II Secretory responses to 10 min of sham feeding in Pavlov pouch dogs with and without perfusion of the duodenal bulb with 0.1 N HCl

Experimental procedure	Number of expts	Secretion in meq acid (mean and range)				
		Control period	Response to 10 min of sham feeding			
		(1 hr)	1st hr	2nd hr	3rd hr	4th hr
		Dog 5				
Controls	3	0.02 (0—0.04)	1.46 (1.40—1.51)	1.64 (1.45—1.88)	0.82 (0.52—1.26)	0.43 (0.14—0.88)
0.1 N HCl in the duodenal bulb for 5 min during the sham feeding procedure	3	0.06 (0.02—0.10)	0.31 (0.11—0.44)	0.97 (0.37—1.40)	0.70 (0.56—0.91)	0.60 (0.28—1.16)
As percentage of means for controls		—	21	59	85	153
0.1 N HCl in the duodenal bulb for 30 min before and 30 min after the start of the sham feeding procedure	3	0.03 (0—0.08)	0.03 (0.03—0.04)	0.01 (0—0.02)	0.09 (0.08—0.12)	0.78 (0.49—1.03)
As percentage of means for controls		—	2	1	11	181
0.1 N HCl in the duodenal bulb for 30 min before and 4 hrs after the start of the sham feeding procedure	3	0.02 (0—0.03)	0.01 (0.01—0.02)	0 (0—0.01)	0.02 (0.01—0.02)	0.02 (0.01—0.03)
As percentage of means for controls		—	1	0	2	5
		Dog 21				
Controls	3	0.09 (0—0.17)	2.64 (2.12—3.11)	1.21 (0.72—1.72)	0.70 (0.23—1.05)	0.49 (0.26—0.61)
0.1 N HCl in the duodenal bulb for 5 min during the sham feeding procedure	3	0.04 (0—0.06)	1.39 (1.14—1.70)	1.53 (1.19—1.93)	0.55 (0.24—0.87)	[illegible] (0.11—0.3[illegible])
As percentage of means for controls		—	53	126	79	69
0.1 N HCl in the duodenal bulb for 30 min before and 30 min after the start of the sham feeding procedure	3	0.01 (0—0.02)	0.38 (0.13—0.86)	1.20 (0.87—1.47)	1.34 (0.82—2.03)	0.80 (0.32—1.12)
percentage of means for controls		—	14	99	191	163
l in the duodenal bulb for 30 efore and 4 hrs after the start sham feeding procedure	3	0.03 (0—0.05)	0.27 (0.12—0.48)	0.03 (0—0.0[illegible])	0.04 (0.03—0.07)	0.02 (0—0.04)
As percentage of means for controls		—	10	2	6	4

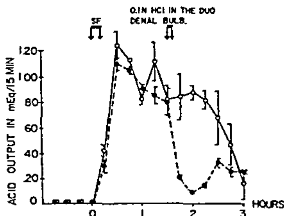


Fig 4 Acid responses to 10 min of sham feeding in a Pavlov pouch dog (22) with (closed symbols) and without (open symbols) perfusion of the duodenal bulb with 0 1 N HCl for 5 min Each symbol represents the mean of 2 expts Vertical bars show the range

bulbar perfusion with 0 1 N HCl was varied Bulbar perfusion for 5 min considerably decreased the acid output, the perfusion was started either simultaneously with (Table II) or after (Fig 4) the sham feeding period Longer periods of bulbar perfusion prolonged the duration of inhibition (Table II)

Series C

The results from series C are presented in Fig 5 Reduction of the intrabulbar pH to 4 produced inhibition of the sham feeding response More pronounced inhibition was obtained when the intrabulbar pH was further reduced Maximal inhibition occurred at pH 1 1—1 4

Discussion

In a series of investigations the pattern of gastric secretory inhibition induced by acid in the duodenal bulb has been studied by using various secretory stimuli Thus, it was shown that irrigation of isolated pouches of the duodenal bulb with 0 1 N solu-

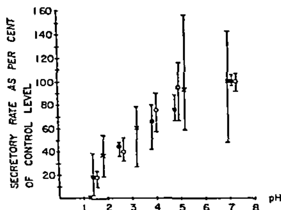


Fig 5 Acid responses to 10 min of sham feeding in 3 Pavlov pouch dogs (5=x, 7=● 18=○) at different pH s in the duodenal bulb Each symbol represents the mean of 3 expts. in one dog Vertical bars show the range pH values refer to the pH of the perfusate

tions of HCl caused pronounced inhibition of secretory responses from gastric pouches to exogenous gastrin (Andersson *et al* 1965 1967, Andersson and Nilsson in press)

In the present study gastric secretion was induced by sham feeding Sham feeding is considered to mimic the cephalic phase of the gastric secretory cycle Sham feeding stimulates the gastric acid secretion by a dual mechanism By synergistic action between direct vagal action and gastrin on the HCl glands these are brought into activity

There is experimental evidence that bulbar inhibition is mediated by a humoral mechanism acting at the HCl glands rather than by a nervous mechanism Thus acidification of totally denervated bulbar pouches inhibited acid responses to exogenous gastrin (Nilsson to be published) The bulbar mechanism has a great efficiency in inhibiting acid responses to gastrin Lowering of the intrabulbar pH to 3—4 abolished acid responses to doses of gastrin which were just enough to cause the HCl glands to secrete in Pavlov pouch dogs (Andersson and Nilsson in press) If the bulbar pH was further reduced the acid responses to even larger doses of gastrin were completely inhibited (Andersson *et al* 1967 Andersson and Nilsson in press)

Vagal activation by sham feeding releases amounts of gastrin from the antrum which per se are too small to cause the HCl glands to secrete (Olbe 1964 a) Since the bulbar mechanism is very efficient in inhibiting acid responses to gastrin (Andersson *et al* 1967, Andersson and Nilsson in press) one would expect that reduction of the intrabulbar pH to about 1 should completely inhibit acid responses to vagal activation in which the vagal excitation is dependent on gastrin to cause the HCl glands to secrete Incomplete inhibition of acid responses to vagal activation might then be due to inability of the bulbar mechanism to influence the direct vagal excitation of the HCl glands

In recent studies it was found that vagal activation by sham feeding for 1 min (Nilsson and Sjodin to be published) and for 10 min (Olbe 1964 b Nilsson and Sjodin to be published) requires gastrin to cause the HCl glands to produce significant acid responses Sham feeding for 30 min however still produced high acid responses in Pavlov pouch dogs which had undergone surgical resection of the antrum and *the duodenal bulb (Nilsson and Sjodin to be published)*

In the present study bulbar perfusion with 0 1 N HCl completely inhibited acid responses to 1 and in some dogs to 10 min of sham feeding whereas inhibition of acid responses to 30 min of sham feeding was less pronounced Since acid responses to prolonged sham feeding (30 min) are less susceptible to bulbar inhibition direct vagal excitation of the HCl glands is probably not suppressed by the bulbar mechanism Instead the bulbar mechanism may inhibit acid responses to vagal activation by interference with gastrin

Together the present and previous results (Andersson and Uvnas 1961 Andersson *et al* 1965 1967 Andersson and Nilsson in press Nilsson in press) from experiments on dogs have given strong evidence for the existence of a mechanism in the duodenal bulb which inhibits gastric acid secretion Short periods of acidification of

the bulbar mucosa are required to produce secretory inhibition and both the present and previous results (Andersson and Nilsson, in press) show that reduction of the pH in the duodenal bulb to pH 3—4 is sufficient to activate the bulbar inhibitory mechanism

This investigation was supported by research grants from Statens Medicinska Forskningsråd B67 14X 88 03, Karolinska Institutet and Torsten and Ragnar Söderbergs Stiftelse

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Demonstration of Adrenergic Receptor Function and Innervation in the Ductus Arteriosus of the Human Fetus

By

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Abstract

Boreus, L O, T Malmfors, D M McMurphy and L Olson *Demonstration of adrenergic receptor function and innervation in the ductus arteriosus of the human fetus* Acta physiol scand 1969 77 316—321

the ductus in neonates

The possible mechanisms for the post-natal closure of the ductus arteriosus have been studied by many (for review see Dawes 1968) Oxygen has been shown to be a strong constricting agent in animal experiments both *in vitro* and *in vivo* Furthermore the association of hypoxia with patency of the ductus is well known On the other hand the proposed roles of chemical mediators like catecholamines acetylcholine 5 hydroxytryptamine and bradykinin are less settled (Born *et al* 1956 Kovalčík 1963 Smith Morris and Assali 1964, Melmon *et al* 1968)

The purpose of the present study was to investigate the possibility of an adrenergic mechanism involved in the closure of the ductus arteriosus in man using tissues obtained from human fetuses The fetal ductus was studied by a combination of two different techniques i e the determination of adrenergic receptor function *in vitro* and the histochemical demonstration of adrenergic nerves by means of the Falck Hillarp fluorescence technique

Material and methods

Fetuses

Twenty-one human fetuses of estimated gestational age from 10 to 24 weeks (crown rump CRL 5 5—20 cm) were obtained from legal abortions Nine of these were used for function experiments nine for histochemical studies and three for both

[1] Trainee of U S Public Health Service Training Grant 5 T01 HD 00166 03

Receptor function experiments

I *Perfusion experiments (7 fetuses)* A polyethylene catheter was introduced into the distal [illegible] ductus The aorta [illegible] main pulmonary [illegible] d 20 ml overflow [illegible] vol% O_2 and 6 5 [illegible] pH of 7 4 Tem [illegible]

The catheter was connected to a perfusion set up with a constant flow rate pump (Watson Marlow H R. Flow Inducer) and a pressure transducer (Statham P 23BB) The perfusion fluid was Tyrode solution delivered from the reservoir The perfusion rate was usually 6 ml/min which gave a base line pressure of about 10—20 cm H_2O Changes in the pressure resulting from the administration of drugs were recorded (Offner Dynograph) Drugs were injected directly into the perfusion system Acetylcholine iodide noradrenaline bitartrate and the alpha adrenergic blocking drug, phentolamine (Regitin) were used All concentrations are given in terms of the salts Fresh drug solutions were made up in distilled water for each experiment. Injection volumes were 0 1—0 3 ml Control injections of distilled water had no effect

II *Spiral strip experiments with ductus arteriosus (2 fetuses) or thoracic aorta (7 fetuses)* Isolated spiral strip preparations of the ductus arteriosus or the thoracic aorta were placed under tension in a 20 ml overflow bath at 37° C, filled from a reservoir of Tyrode solution through which the gas mixture bubbled Cumulative dose effect curves (van Rossum and van den Brink 1963) were determined for noradrenaline and acetylcholine

Histochemical experiments

Tissues were either taken directly to histochemistry or studied after the receptor function experiments had been performed They were [illegible] be quenched in liquid propane cooled by [illegible] aldehyde gas for the histochemical fluore[illegible] and Jonsson 1967, Norberg 1967 Olson [illegible] specific fluorescence of the adrenergic nerves were found in tissues stored for as long as 24 hrs in cold Tyrode solution or in those tissues previously used in the receptor function experiments

Results

Receptor function experiments

I *Perfusion experiments* Acetylcholine (0 1—3000 μg) produced dose dependent contractile responses which were reproducible without fatigue (Figs 1 and 2 The resulting pressures from the highest doses i e 1000—3000 μg were 31—63 cm H O

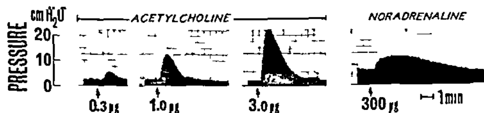


Fig 1 Actual recordings of acetylcholine and noradrenaline induced contractions in the isolated perfused human fetal ductus arteriosus (CRL—10 cm Note the prompt response both drugs and that the effect of noradrenaline is longer lasting and that the dose is

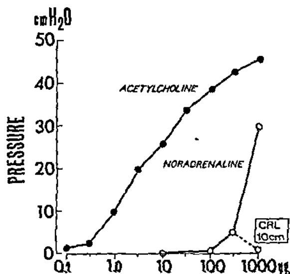
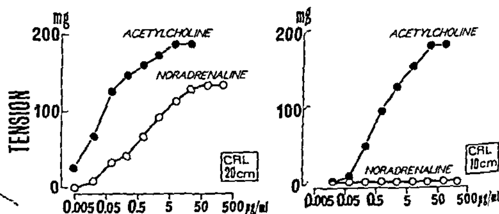


Fig 2 Dose effect curves of acetylcholine (solid circles) and noradrenaline (open circles) in the isolated perfused human fetal ductus arteriosus Injections of acetylcholine could be made in succession without tachyphylaxis or fatigue, but there was a marked degree of tachyphylaxis with noradrenaline This is demonstrated by the two response to 1000 μg When the dose was given immediately after the previous dose (low dotted line) a very low response was obtained but when given after a long wait (3 1/4 hrs), a much higher response resulted

Noradrenaline caused a contraction of the ductus which was associated with a marked degree of tachyphylaxis (Figs 1 and 2) This phenomenon was noted even when the interval between two successive injections was as long as 1—2 hrs and made it difficult to determine a full dose effect curve

In one experiment addition to the perfusion fluid of the alpha adrenergic antagonist, phentolamine (5 μg/ml), blocked the contractile response to noradrenaline completely, but transiently as when the blocker was washed out the response of the ductus returned to the pre blockade level



3 Cumulative dose effect curves of acetylcholine (solid circles) and noradrenaline (open circles) from isolated spiral strip preparations of human fetal ductus arteriosus (left panel) aorta (right panel) Both tissues contract with acetylcholine Ductal tissue responds to noradrenaline so that a dose effect curve can be determined Aorta is not sensitive to noradrenaline

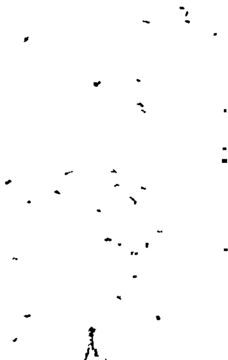


Fig 4

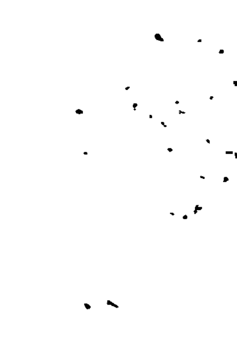


Fig 5

Fig 4 Fluorescence microphotograph of the human fetal ductus arteriosus ×120 Tangential section through the circular muscle layer of the media Numerous fine varicose fluorescent nerve terminals are seen running parallel to the muscle bundles An oblique section through parts of the internal elastic membrane (autofluorescent) at bottom

Fig 5 Fluorescence microphotograph of the junction between the human fetal ductus arteriosus (upper half) and the descending aorta (lower half) ×120 The medial muscle layer of the ductus is transversally sectioned and the varicose nerve terminals appear as white dots The descending aorta lacks fluorescent nerves but contain numerous elastic lamellae visible owing to their weak autofluorescence

II *Spiral strip experiments* In the largest fetus it was technically possible to make a spiral strip preparation of the ductus in order to determine the dose effect relationship for noradrenaline and acetylcholine (Fig 3 In this preparation there was no disturbing tachyphylaxis to noradrenaline

In strips of ascending and descending thoracic aorta acetylcholine produced good prompt contractions so that a dose effect curve could be determined Fig 3 However noradrenaline never produced any contractile response in a wide dose range (0 005—500 μg/ml)

Histochemical experiments

Abundant thin fluorescent fibers with pronounced varicosities were consistently found between the smooth muscle bundles of the media Fig 4 The media was of considerable thickness and the nerves were more frequently found at the

parts of the media. In the youngest fetuses, nerves were in fact found only in the outermost parts of the media. Most nerve fibers ran parallel to the circular muscle bundles. No fluorescent nerve terminals were found in the descending aorta. Elastic lamellae were not found in the ductus except for the internal elastic membrane. This was in contrast to the large number of elastic lamellae found in the descending aorta at the departure of the ductus (Fig. 5).

Discussion

A review of the literature on the role of catecholamines, in particular noradrenaline, in ductal closure disclosed that all experiments had been carried out in animals (fetal lambs and guinea pigs). Results, however, were conflicting in that noradrenaline was found to constrict the fetal animal ductus by some (Born *et al.* 1956 and Kovalčik 1963), but not by others (Smith, Morris and Assali 1964). No reports of experiments in the human fetal ductus arteriosus were found.

Both adrenergic and cholinergic receptor function has been found to be well developed early in human fetal life (Boreus 1967, 1968; McMurphy and Boreus 1968 and unpublished data). The present *in vitro* experiments on the human fetal ductus arteriosus also demonstrate the presence of adrenergic and cholinergic receptor function since a prompt ductal contraction followed the administration of noradrenaline and acetylcholine in a dose dependent fashion. Also, the noradrenaline response could be blocked by an alpha antagonist. As we have shown earlier, acetylcholine causes contraction of many other human fetal tissues, such as ileum, esophagus, trachea, aorta and pulmonary artery. The cholinergic function in ductal closure should therefore be investigated in more detail. Furthermore, it is to be noted that the oxygen tension in these studies was kept constant and high. No attempts were made to elucidate the influence of variations in the oxygen tension on the contractile state of the ductus.

The responsiveness of the human fetal ductus arteriosus to noradrenaline correlates well with the histochemical fluorescence demonstration of noradrenaline nerve terminals in the smooth muscle layer of the media. The sympathetic innervation of blood vessels has been shown to form a plexus of nerves confined mostly to the outer border of the media (Ehinger, Falck and Sporrong 1966; Norberg 1967). However, as shown in this paper, the nerve terminals in the human ductus are not restricted to the outer border of the media but penetrate more deeply into it. Adrenergic nerve terminals in other developing organs from man and other species do not have as pronounced varicosities as those in the ductus arteriosus (Ehinger *et al.* 1968; Olson *et al.* 1969). The appearance of the adrenergic nerve terminals of the ductus arteriosus is more like that of adult tissues and suggests an earlier maturation of the fibers which may play a role in the closure of the ductus.

In the ductus, a noradrenaline contractile response as well as adrenergic nerve terminals were found. On the other hand, in the thoracic aorta neither noradrenaline sensitivity nor fluorescent nerve terminals could be demonstrated. Thus the

results from the receptor function experiments and histochemical studies correlated well In conclusion therefore, the human fetal ductus seems to be prepared with an adrenergic mechanism, which later in neonatal life may contribute to its closure

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Uptake and Distribution of Rubidium-86 and Potassium-43 in Mice and Rats—an Autoradiographic Study

By

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Abstract

Olsson, K. A, R Soremark and K R Wing *Uptake and distribution of rubidium 86 and potassium-43 in mice and rats — an autoradiographic study* Acta physiol scand 1969 77 322—332

The uptake and distribution in mice of rubidium 86 (^{86}Rb) and potassium-43 (^{43}K) was studied using whole body autoradiographic techniques In addition a microautoradiographic study of the distribution of ^{86}Rb in the heads of rats was made The results demonstrate high relative accumulation of ^{86}Rb in hyaline and articular cartilage tendon, forming bone liver pancreas salivary glands thymus spleen and skeletal muscle At one hour post injection the distribution pattern of ^{43}K was similar to but much less differentiated than that of ^{86}Rb [illegible] present results demonstrate high relative accumulation of ^{86}Rb and ^{43}K in hyaline and articular cartilage tendon and forming bone and high ^{86}Rb concentrations in forming teeth and foetal bone and cartilage observations which have not been made in earlier studies The *similarities to and differences from the results of a similar study of the uptake and distribution* of caesium 137 (^{137}Cs) are discussed

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Rubidium 86 (^{86}Rb) has been used as a tracer nuclide for potassium in uptake transfer and dilution studies (Rubino *et al* 1958 Mabille *et al* 1961b Burch and Ray 1958 Henegar Friskey and Preston 1959 Becker 1962 In general the distribution and uptake of this radionuclide appear to be proportional to the distribution of naturally occurring potassium and rubidium (Relman 1956) Renal excretion of [illegible]idium or ^{86}Rb parallels that of potassium or potassium 42 (^{42}K) at about one [illegible]f the potassium excretion rate (Rubino *et al* 1958 Kunin *et al* 1959, Mussa[illegible] [illegible]62) The excretion of ^{86}Rb by sweat and salivary glands is similar to that of ^{42}K [illegible]renson and Burch 1957 Langley and Brown 1961) Tracer studies in mammals and external counting and sampling studies in man have demonstrated significant quantitative differences in the uptake of ^{86}Rb and ^{42}K in a number of tissues Within

a few hrs to a few days after administration ^{86}Rb is less concentrated than ^{42}K in brain urine, bone and plasma At the same time, these two radionuclides are nearly equally concentrated in erythrocytes and skeletal muscle while more ^{86}Rb than ^{42}K is taken up in the viscera (Kilpatrick *et al* 1956, Tyor and Eldridge 1956) It has also been shown that a sizable discrepancy exists between the rubidium and potassium spaces as determined by dilution experiments (Henegar *et al* 1959, Relman 1956, Kilpatrick *et al* 1956 Martin and Walker 1958)

Rubidium can to some extent act as a physiologic replacement for potassium in some tissues while caesium is less suitable as a substitute In several tissues, these three alkali metals are not taken up in the same proportions as are found for the organism as a whole (Relman 1956) The possibility exists that rubidium and/or caesium play physiologic roles, indirectly by competitive inhibition of a potassium effect or directly by being essential to some process The first step in the investigation of this possibility is to compare the relative uptake of radioisotopes of these three elements in the various organs and tissues of the body

In the present investigation, the uptake and distribution of ^{86}Rb in mice was studied by means of whole body autoradiography A second series of whole body autoradiograms was made from mice which had been administered ^{43}K in order that comparisons might be made with the ^{86}Rb data of the present study and the caesium 137 (^{137}Cs) data of Nelson *et al* (1961) In addition a series of microautoradiograms was made from sections of the heads of young rats which had been administered ^{86}Rb These autoradiograms were used to provide more detailed information on ^{86}Rb uptake with special emphasis on erupted and unerupted teeth and the surrounding hard tissues

Materials and Methods

^{86}Rb and ^{43}K were produced in a neutron reactor (The Swedish Atomic Energy Co Studsvik Nykoping) ^{86}Rb was obtained by irradiating RbCl in a thermal neutron flux of 1.4×10^{12} neutrons cm^{-2} sec^{-1} Carrier free ^{43}K was obtained by irradiating enriched $^{43}CaCO_3$ with thermal neutrons according to a method by Forberg (1962) The radionuclides thus obtained were diluted with physiologic saline solution before injection One to two μCi per gram body [illegible]

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For micro autoradiography 8 male rats of the Sprague-Dawley strain 9 days old were injected intraperitoneally with ^{86}Rb Four rats were sacrificed 1 day and 5 days after the single injection The rats were lightly anesthetized with ether and then sacrificed by decapitation The heads were immediately immersed in the cold solution

For whole body autoradiography twenty micron sagittal section were taken at various levels through the frozen specimens using a sledge microtome The sections were mounted on adhesive tape The sections on tape were placed in contact with Kodirex X ray films during the exposure period For micro autoradiography five micron serial sagittal sections [illegible] ade

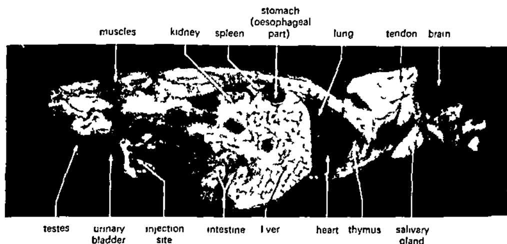


Fig 1 A whole body autoradiogram from a section of a male mouse 1 hr after i p injection of ^{86}Rb White areas correspond to tissues or organs with high relative concentrations of radio rubidium This autoradiogram is also representative for the female mouse which was sacrificed at 1 hr post injection

through the rat heads and were mounted on adhesive tape The sections of the rat heads were then placed in permanent contact with Ilford G5 autoradiographic plates After the exposure development and fixation of the G5 emulsions the sections were stained with haemalum and eosin The whole body and micro autoradiographic techniques used have previously been described by Hammarstrom (1966) and others

Results

Rubidium 86 (whole body and micro-autoradiography)

The pattern of accumulation of ^{86}Rb in the tissues of mice *relative to that in skeletal muscle* remained relatively constant from 1 hr through 5 days post-injection except in the liver, foetus and lens where the accumulations of ^{86}Rb increased, and in the blood lung kidney urine and placenta, where the relative activities decreased between 1 and 5 hrs post injection (Fig 1 and 2) Despite the different routes of administration no differences were seen between the accumulation patterns in the male and female mice The following descriptions unless otherwise stated apply to all sacrifice times under investigation and to both males and females

A general comparison of the uptake of ^{86}Rb seen in the micro-autoradiograms of the heads of rats with that in the corresponding tissues in the whole body autoradiograms of mice revealed no discernable differences In the following descriptions therefore the information obtained from the micro autoradiograms will be used to supplement that from the whole body autoradiograms despite the fact that different species were used

Circulatory and reticulo endothelial systems ^{86}Rb was rapidly lost from blood such that, by 1 hr post-injection the activity in blood was among the lowest in the body The micro-autoradiograms showed some activity remaining in the erythrocytes

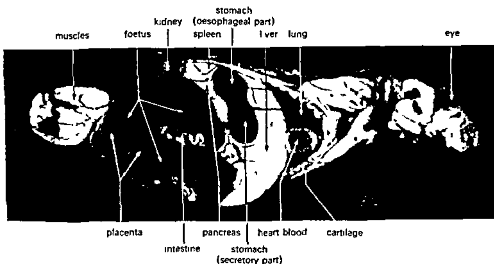


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Blood vessel walls showed a lower accumulated activity than that in blood Heart muscle showed a moderate uptake of ^{86}Rb, significantly more than in blood and less than in skeletal muscle

The concentrations in thymus and spleen were approximately the same as in the skeletal muscle The liver accumulated more ^{86}Rb than any of the other viscera and more than skeletal muscle, the relative concentration increased markedly between 1 and 5 hrs post-injection No differentiation of accumulation of ^{86}Rb in liver parenchyma and the reticulo-endothelial elements could be made Bone marrow and lymph nodes had low ^{86}Rb concentrations

Respiratory system The lung tissue including the bronchi showed a low uptake of ^{86}Rb, appearing to be approximately equal to and decreasing with that in blood It must be remembered however, that the lung tissue represents a very small portion of the area of the lung and the actual ^{86}Rb concentration must be much higher than it appears The uptake in the respiratory mucosa was relatively low A very heavy accumulation occurred in the hyaline cartilage of the tracheal rings and nasal conchae

Muscle, bone, skin, and associated tissues In skeletal muscle including that of the tongue, the accumulations of radiorubidium was among the highest in the body being lower than those in hyaline and articular cartilage tendons forming bone and liver The tendons showed more activity than their associated muscle and much more than cortical bone

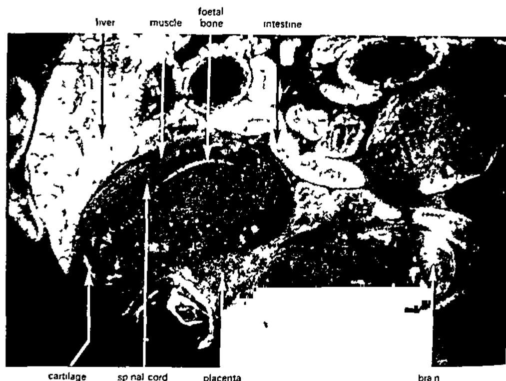


Fig 3 An enlargement of a portion of a whole body autoradiogram showing the distribution of ^{86}Rb in the abdomen of a pregnant mouse 5 days after an i v injection Note the high relative uptake in cartilage and bone and the even distribution of low activity in the soft tissues in the foetus

Bone of all types showed a remarkably low uptake except at points of rapid growth The micro-autoradiograms showed that the trabecular bone of the mandible had a higher concentration than the cortical bone and the blood

Hyaline cartilage of the ribs and articular cartilage as well as the hyaline cartilage of the trachea and nasal conchae showed the greatest concentration of ^{86}Rb of all tissues of the body The fibrocartilage of the intervertebral discs, on the other hand contained very little radiorubidium

Skin and subcutaneous fat showed low concentrations of radiorubidium In the hair follicles the uptake of ^{86}Rb was also low In the central parts of the hair shafts however, a moderate amount of activity had accumulated

Teeth As in compact bone the uptake of radiorubidium in dentin and enamel is low However the micro-autoradiograms demonstrated high concentrations in developing unerupted teeth relative to the surrounding hard tissues (Fig 4) A high relative concentration was seen in the odontoblast and ameloblast regions Very little activity was visible in the dental pulp



Fig 4 An enlargement of a small portion of a micro-autoradiogram of the head of a male rat showing the distribution of ^{86}Rb in a developing molar tooth 1 day after an i p injection The region of high relative activity is here seen as a grey shadow superimposed on the regions of growth in the enamel and dentin

Digestive tract The mucosal walls of the mouth oesophagus and the oesophageal portion of the stomach contained very little activity while the secretory part of the stomach and the intestinal walls showed a moderate concentration or nearly as much as that in skeletal muscle The gastric and intestinal contents had much less activity than the gastric and intestinal mucosa

Of the associated viscera liver showed the greatest accumulation of ^{86}Rb while the gall bladder showed very little activity Pancreas and skeletal muscle activities were approximately equal In the salivary glands, a moderate concentration of ^{86}Rb was observed at all post injection periods being approximately the same as in skeletal muscle

Urogenital system By 1 hr moderate activity had accumulated in the urine By 5 hrs, the activity in urine had markedly decreased The activity in the kidney at 1 hr was about equal to that in skeletal muscle From 5 hrs through 5 days the ^{86}Rb concentration decreased to a level below that of skeletal muscle No differentiation was ever seen between cortical and medullary accumulation in the kidney

Testis showed a low uptake of ^{86}Rb

Lactating glands also showed a low uptake of ^{86}Rb

The vaginal mucosa had a moderate accumulation of ^{86}Rb No ovaries could be identified In the placenta the accumulation was low being approximately equal to and decreasing with that in blood

Foetus By 1 hr after the injection activity was visible in the foetal tissues The relative accumulation increased with time Within the foetus (Fig 3), a relatively marked accumulation was seen in cartilage and bone while radiorubidium was evenly distributed in all the soft tissues No activity was visible in the amniotic fluid

Nervous system The central nervous system showed a low, relatively rapid accumulation of ^{86}Rb No differential uptake was seen in the tissues and fluids of the CNS

The lens of the eye accumulated radiorubidium slowly At 1 hour only a narrow zone in the periphery of the lens had any activity At 1 day, the activity zone was much broader but did not become any broader at longer post injection periods The micro-autoradiograms also demonstrated a low uptake in the various tissue layers of the eye bulb The vitreous body showed a somewhat higher accumulation than the other tissues and fluids of the eye The lacrimal glands showed a low concentration

Potassium 43 (whole body autoradiography)

The distribution pattern in mice of radiopotassium at 1 hr post injection was similar to that for radiorubidium (compare Fig 5 and 1) There were, however a few significant differences The distribution of ^{43}K was much more even than that of ^{86}Rb Relative to skeletal muscle accumulation hyaline and articular cartilage tendon and liver showed lower accumulations of ^{43}K than ^{86}Rb while the converse was seen in heart muscle lung, bone and brain No comparisons at later post injection times could be made

Discussion

Due to an elegant production procedure developed by Forberg (1962) ^{43}K can now be made available carrier free in sufficient amounts for biological experiments A comparison of the radiophysical properties of ^{43}K and the commonly used ^{42}K

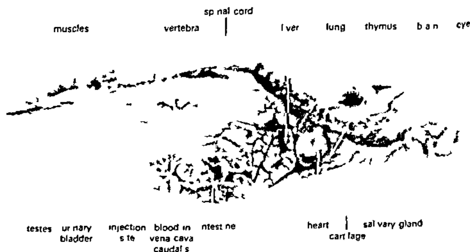


Fig 5 A whole body autoradiogram from a section of a male mouse 1 hr after an ip injection of ^{43}K Note the more even distribution as compared with that of ^{86}Rb at 1 hr (Fig 1)

reveals that ^{43}K is more suitable for autoradiography because of its lower energy beta radiation and longer half life 22.4 versus 12.5 hours (Radiological Health Handbook 1960) Other potassium radioisotopes are not suitable for most biological studies The radiation properties of ^{86}Rb are far from ideal for autoradiography Thin sections and emulsions should be used in order to optimize the resolution in autoradiograms made with these radionuclides

Mice were used for the whole body autoradiographic preparations because of the small size of the adults including the pregnant females and the necessity to minimize problems in sectioning calcified tissues Young rats were used for preparations for microautoradiography of the head region because the larger size permitted easier tissue recognition The fact that the rats were young made it possible to view both unerupted and erupted teeth and also reduced the problems in sectioning calcified tissues

The pregnant female mice were injected intravenously rather than intraperitoneally (which is easier and more certain) in order to avoid injection into the amniotic sacs Comparison of these animals with the males which were injected intraperitoneally reveals no differences in accumulation of ^{86}Rb even as early as 1 hr post injection Unfortunately the amount of ^{43}K available at this time did not permit us to extend this part of the study to include sacrifice times later than 1 hr post injection

Autoradiograms from serial sagittal sections from each mouse and rat were observed in compiling the data No single section contained all of the tissues and organs of interest to the present study The descriptions of the relative concentrations therefore include many which do not appear in the necessarily limited number of autoradiograms presented in Fig 1—5 The radionuclide concentrations in skeletal muscle was used as the reference in all comparisons because samples of skeletal muscle were present in every section and because of the attention which has earlier been paid to the concentrations of these radionuclides in skeletal muscle

One of the most striking observations in the study of the uptake and distribution of ^{86}Rb in mice is the rapidity with which the accumulations of activity in the various tissues and organs come into equilibrium Changes in the concentrations relative to that in skeletal muscle at sacrifice times later than 1 hr post injection occurred in only a few tissues and organs Significant continued increases in activity were observed only in liver foetus and lens Even in these an equilibrium had been achieved by 5 hrs post injection Those tissues and organs which showed decreased relative concentrations of ^{86}Rb between one and 5 hrs post injection are those whose activities would most likely be directly related to the serum activity which must continue to decrease due to excretion and continued uptake by those tissues which are slow to equilibrate Thus kidney and urine concentrations logically follow that of serum Since only small amounts of ^{86}Rb were found in red blood cells the whole blood concentration would tend to follow that of serum The lungs and placenta which contain very much blood in comparison to other viscera might be expected to contain ^{86}Rb in concentrations more or less proportional to that in blood From 5 hrs to

5 days post injection, the concentrations of ^{86}Rb in all tissues and organs appear to maintain a relatively constant relationship to one another, decreasing slowly with increasing time post injection It would appear that none of the tissues irreversably accumulated ^{86}Rb but this remains to be confirmed in quantitative studies

A comparison of the present results with quantitative studies on the uptake of ^{86}Rb in selected tissues of rabbits (Kilpatrick *et al* 1956) and rats (Mabille *et al* 1961a) shows close agreement Mabille *et al* have shown that within 1 day post injection blood had one of the lowest ^{86}Rb concentrations (assuming the whole blood concentration to be an average of those in plasma and red blood cells) with only brain bone and skin being lower Skeletal muscle lungs intestines kidney and heart showed similar concentrations which were above that in blood Liver showed the highest concentration of the tissues studied Except for the concentrations in brain and red blood cells which increased during the first 5 days post injection, the ^{86}Rb content of these tissues decreased uniformly in a parallel fashion through the thirteenth day post injection (Mabille *et al* 1961a)

Neutron activation analysis studies have confirmed the results of earlier investigations which demonstrated that only moderate amounts of rubidium are present in hard tissues (Soremark 1964, Brudevold and Soremark 1967) In addition it has been shown that the dentin and enamel of unerupted teeth are exceptional among the hard tissues studied Unerupted teeth have approximately ten times as much rubidium as erupted teeth or concentrations which lie within the range of those in muscle and viscera Erupted teeth have relatively low rubidium concentrations similar to those of other hard tissues (Lundberg Soremark and Thilander 1965a Lundberg Soremark and Thilander 1965b)

In the present study ^{86}Rb is seen to be accumulated in very small amounts in bone as might be expected from the above data on rubidium concentrations However there were regions within the bone which were very high in ^{86}Rb concentration These were the regions of most active growth and those where tendons attached In the study of ^{43}K distribution 1 hr after injection even greater relative concentrations of ^{43}K than ^{86}Rb were observed in forming bone and in cortical bone in the regions of muscle tendon attachments High relative concentrations of ^{86}Rb were also present in foetal bone In unerupted teeth all regions of active enamel or dentin formation were observed to accumulate high concentrations of ^{86}Rb Since bone as a whole and erupted teeth have relatively low rubidium concentrations it would be expected that these accumulations of ^{86}Rb must be displaced during the mineralization process or perhaps the maturation of the matrix It could be that these high ncentrations represent non selective accumulation of positively charged atoms in e matrix before mineralization begins It is also possible that potassium and/or bidium play an active role in matrix formation maturation or in the beginning stages of mineralization

In a review of the literature Relman (1956) made comparisons of the distribution and uptake of potassium rubidium and caesium in mammalian tissues The distribution patterns of naturally occurring potassium and rubidium are very similar

High concentrations of both elements are found in erythrocytes muscle and viscera and low concentrations in plasma and bone It was also noted that the concentration ratios of rubidium to potassium and of caesium to potassium in animal tissue are much greater than those in the earth s crust indicating that at least some tissues can distinguish among these three alkali metals and take up caesium and rubidium in preference to potassium (Relman 1956)

The results of the present study show that the tissues of greatest accumulation of ^{86}Rb and ^{43}K are hyaline and articular cartilage This is in agreement with the distribution pattern of ^{137}Cs as described by Nelson *et al* (1961) In comparing the relative accumulation of ^{137}Cs in hyaline cartilage with the high relative accumulation of sodium 22 (^{22}Na) in the same tissues (Huggert *et al* 1961) Nelson *et al* (1961) speculated that the accumulation might be non specific in nature and similar to the action of an ion exchanger If this were so one would expect the four ions to be taken up preferentially Cs>Rb>K>Na (Samuelson 1952) An autoradiographic comparison of the accumulation of these four radionuclides (^{137}Cs from Nelson *et al* 1961 ^{86}Rb and ^{43}K from the present study and ^{22}Na from Huggert *et al* 1961) in the cartilage of the nose trachea ribs and articular surfaces relative to the accumulation of each in skeletal muscle at 1 hr post injection shows some variation from this preferential pattern but does not give sufficient evidence to either confirm or refute this hypothesis

Other organs and tissues were seen to vary in their relative uptake of potassium rubidium and caesium (^{137}Cs from Nelson *et al* 1961) The following comparisons are all based on the concentrations of the nuclides relative to their concentrations in skeletal muscle Heart muscle appeared to take up more ^{43}K and ^{137}Cs than ^{86}Rb Liver showed a higher relative uptake of ^{86}Rb than either ^{43}K or ^{137}Cs The intestines thymus and salivary glands accumulated more ^{137}Cs than either ^{86}Rb or ^{43}K Relative to skeletal muscle concentrations in the dam more ^{137}Cs than ^{86}Rb appeared in the foetus (not studied for ^{43}K)

The necessarily limited number of tissues which can be studied quantitatively in investigations such as those of Kilpatrick *et al* (1956) and Mabille *et al* (1961a) serves to emphasize the value of whole body autoradiography as a method for surveying tissues of interest for further quantitative work Further some differential uptake patterns such as that of ^{86}Rb in bone may be missed in quantitative studies on whole organs The whole body autoradiographic data of the present study and that of Nelson et al (1961) indicate that it would be of great interest to study quantitatively the nature of the uptake of potassium rubidium and caesium in various cartilagenous tissues tendon different kinds of bone the dentin and enamel of forming and erupted teeth salivary glands reticuloendothelial tissues placenta and foetal tissues including cartilage and bone The uptake of potassium rubidium and caesium in these tissues has received little attention to date

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Oxygen Uptake in Skeletal Muscle of the Anesthetized Dog during Sympathetic Vasodilatation

By

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Abstract

Bolme, P and J Novotný *Oxygen uptake in skeletal muscle of the anesthetized dog during sympathetic vasodilatation* Acta physiol scand 1969 77 333—343

Oxygen uptake in dog skeletal muscle was studied upon activation of the sympathetic cholinergic vasodilator nerves, upon reflex inhibition of the peripheral vasoconstrictor nervous tone and as a response to i a acetylcholine infusion Oxygen uptake was calculated from the arterio-venous oxygen saturation and the blood flow Stimulation of the hypothalamic vasodilator area or of the sympathetic lumbar chain, after adrenergic nervous blockade, produced an increased blood flow and a rise in calculated oxygen uptake in the muscle In most animals the oxygen uptake returned to, or went slightly below, pre stimulatory levels after an initial increase for about 30 sec However, in some experiments the oxygen uptake was elevated during the whole period of vasodilatation Reflex inhibition of vasoconstrictor tone was produced in cross-circulation experiments by stimulating the carotid sinus nerve of the recipient dog When the [illegible]

are dilated

The sympathetic cholinergic vasodilator nerves are considered to innervate pre capillary resistance vessels in skeletal muscle (Folkow Mellander and Öberg 1961 Rosell and Uvnäs 1962, Renkin and Rosell 1962 a, Bolme and Fuxe 1967) Eliasson *et al* (1951) and Eliasson, Lindgren and Uvnäs (1952) assumed on the basis of experiments in anesthetized cats and dogs that the sympathetic vasodilator nerves were activated to prepare the muscles for sudden effort This view was further supported by Abrahams, Hilton and Zbrożyna (1960 1964) who made experiments in anesthetized and conscious cats and suggested that the vasodilator nerves are activated in defence reactions

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Recently, further studies have been reported concerning the significance of the sympathetic vasodilator nervous mechanism. On the basis of experiments where the vasodilator nerves were activated in conscious dogs, both electrically in the hypothalamus (Bolme *et al.* 1967) and as conditional reflex to exercise (Bolme and Novotný 1969) it was further claimed that the vasodilator nerves are activated prior to situations when an increased muscle blood flow is suddenly required. It therefore appeared of interest to study the transcapillary exchange of oxygen following sympathetic vasodilator nerve stimulation in order to further elucidate the physiological function of these nerves.

Previous studies concerning the effect of sympathetic vasodilatation on the transcapillary exchange in muscle have been controversial. Hyman *et al.* (1959) reported an unchanged or even decreased exchange of I^{131} while Barlow and Walder (1965) and Bolme and Edwall (1968) found an enhanced disappearance of radioisotopes from a skeletal muscle depot during sympathetic vasodilatation. Rosell and Uvnäs (1962), using cats, found an initial increase in oxygen uptake followed by a decrease during sympathetic cholinergic vasodilatation. Considering these apparently conflicting data it seemed of interest to reexamine the problem in the dog.

In the present study we report the effects of sympathetic cholinergic vasodilatation produced by hypothalamic and sympathetic lumbar chain stimulation on the oxygen uptake in dog skeletal muscle. For comparison we also studied the oxygen uptake during vasodilatation produced by reflex inhibition of vasoconstrictor nervous tone and by intra arterial acetylcholine infusion.

Methods

The experiments were performed on 23 mongrel dogs of both sexes weighing 6—18 kg. Most dogs were premedicated with morphine (10 mg/kg s.c.) and anesthetized with a chloralose

the carotid or from the femoral artery by a Statham pressure transducer (P 23 AC).

The relative venous oxygen saturation ("reading value") was continuously recorded by directing the venous outflow from the muscle through a cuvette (volume 0.5 ml) connected to a reflection oximeter (CC-oximeter MO 3, Kipp & Zonen, Delft, Holland). The blood was ...rned to the animal via the femoral vein. All recordings were made on a Grass polygraph ...el 5 C.

To determine the arterial hemoglobin O_2-saturation arterial samples were taken frequently ...ring the exp ... collected under glycerin ... prevent ox ... d in ... ve measurem ... the the same way ... blood samples was then measured using a Brinkman hemoreflector (MO 1—550, Kipp & Zonen, Delft, Holland). The hemoreflection method was previously calibrated against a spectrophotometric method (Drabkin 1950). The O_2-saturation values of the venous samples were plotted against their respective "reading values" from the CC-oximeter. In this way the continuous O_2-saturation readings were made quantitative over the whole scale (for details see Zijlstra and Mook 1962).

The hemoglobin content of the blood samples was measured by a photometer (Linson Jr, AB Ljungberg & Co, Stockholm, Sweden).

The oxygen uptake was determined by multiplying the blood flow (ml/100 g muscle) by the arterio-venous difference in hemoglobin oxygen saturation and the O_2-capacity of the [illegible]

[illegible]

was kept constant (about 2 ml) and was compensated for in the oxygen uptake determinations In the continuous oxygen saturation measurement there was no time loss due to mixing of blood in the cuvette when blood of different saturations passed the system This was tested in model experiments by perfusing blood saturated to different degrees, through the cuvette and continuously recording the O_2-saturation

The sympathetic cholinergic vasodilator nerves were activated by electrical stimulation of hypothalamic pathways via unipolar, 0 45 mm gauge stainless steel electrodes, stereotactically oriented into the appropriate area The stimulus currents were supplied by a Grass model S 4 D stimulator and consisted of rectangular pulses of 2 msec duration, frequencies of 40—80 imp/sec, and intensities of 1.5—5 V In some experiments the vasodilator nerves were activated by electrical stimulation of the transected sympathetic chain through a bipolar silver electrode at the L4—L5 level In these experiments the vasoconstrictor response was blocked with dihydroergotamine (0 1 mg i.a) or by pretreatment of the dog with reserpine for two days (0 5 mg/kg i m daily) The stimulus currents used had a duration of 2 msec, frequencies of 1—10 imp/sec and intensities of 5—8 V

In cross circulation experiments the isolated muscle was perfused with arterial blood from [illegible]

[illegible]

and intensities of 2—6 V

Acetylcholine chloride (1 µg/ml calculated as the base) was infused manually in such a way as to mimic the blood flow increases produced by activation of the sympathetic cholinergic nerves or by inhibition of the vasoconstrictor nervous tone Atropine (0 1—0 2 mg i a.) was given to verify the nature of the vasodilatation elicited on hypothalamic and sympathetic chain stimulation

The i a injections or infusions were given into a previously-cannulated branch of *A poplitea* or through a side arm of the arterial drop recorder when used

Results

Activation of the sympathetic cholinergic vasodilator nerves

In 13 dogs the muscle blood flow was increased by stimulating vasodilator pathways in the hypothalamus The blood flow effect could be blocked or reduced by atropine (0 1—0 2 mg i a) A tachycardia and a slight increase in arterial pressure were also observed The effect on the venous O_2 saturation varied somewhat between the animals However in the majority of the experiments (8 out of 13) stimulation led to a gradual rise in venous O_2 saturation i e to a decreased arterio-venous O_2-difference This effect persisted for one to four minutes after the stimulation had ceased and the circulatory effects had disappeared

A representative experiment is shown in Fig 1 The stimulation produced a rise in arterial pressure The blood flow increased immediately from 5 4 to 12 ml/min but the venous O_2-saturation did not change until 15 sec[1] after the onset of the stimulation Consequently there was an initial rise in calculated oxygen uptake After 30 sec of stimulation however the venous O_2 saturation had increased from 69 to 81 per cent leading to a return of the calculated oxygen uptake to the prestimulatory level A further drop in the calculated oxygen uptake was observ

[1] The time values given are corrected for the dead space of the tub

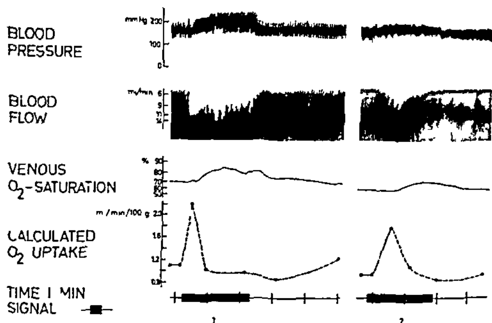


Fig 1 Dog 6 kg Morphine Chloralose M gastrocnemius 28 g Effect of stimulation of vasodilator pathways in the hypothalamus
1 Stimulation with 1 5 V 2 msec 40—80 imp/sec
2 The same as 1 after administration of atropine 0 2 mg i a

ing the remaining period of enhanced blood flow When the stimulation was stopped the blood flow rapidly returned to resting values but the venous O saturation remained at a supra normal level for about two minutes Thus during this period the calculated oxygen uptake was still below prestimulatory levels The effect on the blood flow and oxygen uptake caused by the stimulation was although not abolished considerably reduced by atropine (0 2 mg i a indicating that sympathetic cholinergic fibres had been activated (Fig 1 2)

In five of the 13 dogs stimulated in the hypothalamus the results differed somewhat from those described in Fig 1 In these dogs stimulation produced enhanced muscle blood flow and a rise in arterial pressure However the venous O_2-saturation not much elevated and this led to an increased calculated oxygen uptake during the whole stimulation period The increased oxygen uptake could hardly be due to muscular movements as the results were the same even after the administration of the neuromuscular blocking agent gallamine iodide (Flaxedil® One such experiment is shown in Fig 2 In the left panel the hypothalamic stimulation led to an arterial pressure rise and an increased muscle blood flow The O_2-saturation did not change very much and thus the calculated oxygen uptake was elevated during the whole period of blood flow rise The fact that sympathetic cholinergic vasodilator nerves had been activated is shown by *the blocking effect of atropine (right panel)*

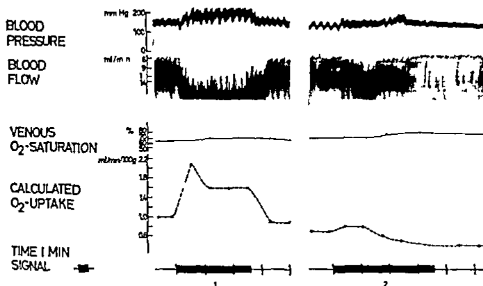


Fig 2 Dog 13 kg Morphine Chloralose M gracilis 49 g (From the same experiment as Fig 3) Effect of stimulation of vasodilator pathways in the hypothalamus
1 Stimulation with 3—5 V 2 msec 50—80 imp/sec
2 The same as 1 after administration of atropine 0 2 mg i.a.
Note that the calculated oxygen uptake before atropine was elevated during the whole period of vasodilatation

In order to get as pure cholinergic vasodilatory effects as possible, the sympathetic lumbar chain was stimulated in five dogs The vasoconstrictor nervous response was blocked by dihydroergotamine (Orstanorm® 0 1 mg i a.) or in two dogs by pretreatment for two days with reserpine (Serpasil® 0 5 mg/kg i.m daily) In all experiments sympathetic chain stimulation produced the same effects an atropine sensitive muscle vasodilatation and an initially increased oxygen uptake followed by a return to resting values after 20—30 sec Fig 4 illustrates these effects dihydroergotamine had been given in this experiment Following stimulation with either 1 imp/sec or 5 imp/sec there was a drop in venous O_2-saturation together with the initial blood flow rise During this initial period the calculated oxygen uptake increased three to four fold After 20—40 sec the venous O_2 saturation started to rise and the oxygen uptake returned to resting values After the stimulations the oxygen uptake was even somewhat decreased the most pronounced decrease being after 5 imp/sec which had produced the greatest blood flow rise Atropine (0 15 mg i a) abolished the blood flow effect caused by the stimulation There was also no change in the venous O_2 saturation in response to the sympathetic chain stimulation after atropine

Acetylcholine infusion

As a regular finding acetylcholine produced changes in blood flow and v O_2-saturation which were similar to those usually seen af

BLOOD PRESSURE mmHg 200 100 0

BLOOD FLOW ml/min 6 9 11 14

VENOUS O_2-SATURATION % 90 80 70 60

CALCULATED O_2-UPTAKE ml/min/100g 1.8 1.4 1.0 0.6 0.2

TIME 1 MIN SIGNAL

Fig 3 Dog 13 kg Morphine Chloralose M gracilis 49 g (From the same experiment as Fig 2)
At signal acetylcholine 1 µg/ml infusion (total dose 1 µg)
Note the initial increase in calculated oxygen uptake followed by the decrease

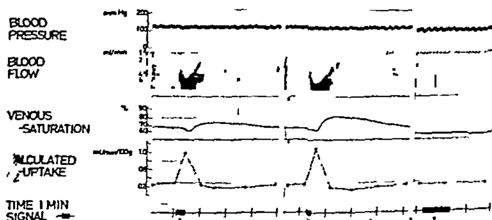
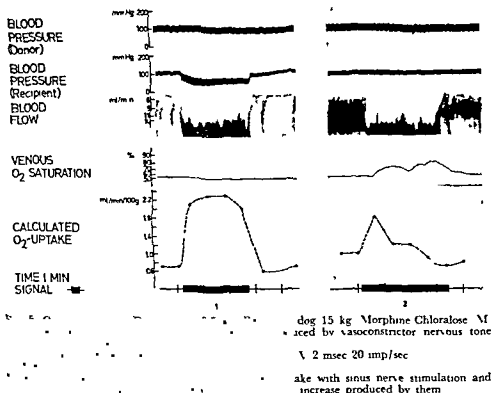


Fig 4 Dog 12 kg Pentobarbital M gastrocnemius 51 g Effect of stimulation of lumbar sympathetic chain D hydroergotamine 0 1 mg i a had been previously given to block the vasoconstrictor nervous response
1 Stimulation with 5 V 1 msec 1 imp sec
2. Stimulation with 5 V 1 msec 5 imp sec
3 Atropine 0 15 mg i.a



... dog 15 kg Morphine Chloralose M
... uced by vasoconstrictor nervous tone

... 2 msec 20 imp/sec

... ake with sinus nerve stimulation and
... increase produced by them

thetic vasodilator nerves This is illustrated in Fig 3 (see also Fig 5 2) The blood flow increased rapidly, but the venous O_2-saturation did not change until 10—15 sec later Thus, the calculated oxygen uptake was initially increased However, during the rest of the infusion the venous O_2-saturation gradually increased in spite of a constant blood flow Thus, the calculated oxygen uptake soon returned towards resting values After about two minutes of dilatation the calculated oxygen uptake was even decreased, an effect which persisted for five minutes after the acetylcholine infusion had ceased even though the blood flow had returned to resting values after about 30 sec After atropine (0 1—0 2 mg i a) no effect in blood flow or oxygen uptake was elicited by acetylcholine

Inhibition of the vasoconstrictor nervous tone

In six experiments muscle vasodilatation was produced by stimulating the carotid sinus nerve on one side, thus reducing or eliminating the prevailing sympathetic vasoconstrictor nervous tone In response to the sinus nerve stimulation the arterial pressure decreased markedly Therefore, in order to keep the perfusion pressure constant during the stimulation, the isolated muscle was perfused with a

from a donor dog Under these circumstances the inhibition of vasoconstrictor tone in all experiments led to an increased oxygen uptake during the period of enhanced muscle blood flow The magnitude of the rise in oxygen uptake was correlated to the blood flow effect a large increase in blood flow was paralleled by a large increase in oxygen uptake

A representative experiment is shown in Fig 5 1 Sinus nerve stimulation produced a three fold increase in blood flow but the venous O_2 saturation was unchanged or even slightly decreased This resulted in a substantial rise in the calculated oxygen uptake, from 0 7 to 2 3 ml O_2/min/100 g muscle The reaction on acetylcholine infusion was the usual one (Fig 5 2) The blood flow was increased to the same extent as by the sinus nerve stimulation However, the calculated oxygen uptake was only initially increased and went down to, and even below, resting levels during the dilatation

Discussion

The present study is a part of a systematic investigation of the physiological role of the sympathetic cholinergic vasodilator nerves *In the experiments reported here we* have increased the blood flow in resting muscles by vasodilator nerve stimulation and compared the effects with those following other types of vasodilatation Under our experimental conditions the resting muscle in the anesthetized dog had the capacity to increase its oxygen uptake during vasodilatation Whether or not this was due to muscular anoxia present in the anesthetized but not in the conscious animal cannot be judged from our experiments

Vasodilatation in dog skeletal muscles due to sympathetic vasodilator nerve activation resulted in an initial increase in the calculated oxygen uptake Rosell and Uvnas (1962) have previously reported similar results in cats The increase in the calculated oxygen uptake observed initially during the vasodilatation might reflect an increased transcapillary oxygen exchange However with the method used in our experiments it is not possible to determine if changes in capacitance function of the muscle blood vessels contributed to the effect If highly deoxygenated blood washed out from the veins during the initial period of vasodilatation mixed with blood which had just passed the capillaries this would interfere with our calculations of oxygen uptake (*cf* Mertens and Rein 1938, Kramer and Schafer 1939 Thus [illegible]ther the observed initial increase in calculated oxygen uptake was due to a real [illegible]apillary O uptake or not cannot be deduced from our experiments

During sympathetic cholinergic vasodilatation the calculated oxygen uptake had usually returned to prestimulatory levels after 20—30 sec In many dogs the oxygen uptake was then further decreased an effect persisting after cessation of the stimulation when the blood flow had returned to resting levels This decrease in the oxygen uptake might reflect an autoregulatory closure of precapillary *sphincters* leading to a reduced capillary surface area Sympathet[illegible] vasodilatation of the arterioles will lead to an increased transmural pressure a[illegible] apillary sphincter

level causing the sphincter muscles to contract (Djojosugito *et al* 1968) Rosell and Uvnas (1962) discussed these mechanisms in detail as a possible explanation of the decrease in oxygen uptake

The effect on oxygen uptake following acetylcholine infusion was similar to that usually observed following sympathetic cholinergic vasodilator nerve stimulation These results indicate that acetylcholine and the vasodilator nerves exert their precapillary dilatory effect mainly on the resistance vessels at least after the initial period of vasodilatation The rather rapid return in calculated oxygen uptake after the initial increase suggests that the precapillary sphincters are influenced to a much smaller extent As was the case following sympathetic vasodilatation a slight de crease in calculated oxygen uptake was seen after the initial increase during acetyl choline infusion indicating a closure of precapillary sphincters

Djojosugito *et al* (1968) reported different effects on capillary filtration coef ficient (CFC) upon sympathetic vasodilatation and following acetylcholine infusion in the cat Those authors observed an increased CFC in response to acetylcholine and a decreased CFC during sympathetic vasodilatation Those experiments cannot be *directly compared with ours since the studies were performed in different species* However Djojosugito *et al* (1968) reported that during acetylcholine infusion CFC did not increase by more than 50 per cent whereas during maximal metabolic vaso dilatation 3—4 fold increases are observed (Kjellmer 1964) Thus the experiments by Djojosugito *et al* (1968) indicate that acetylcholine produces only moderate ef fects on the precapillary sphincters in skeletal muscle of the cat at least with doses giving blood flow increases comparable to those produced by vasodilator nerve stimu lation

It must be emphazised that acetylcholine infusion was used in our experiments only as a standard procedure to produce blood flow increases of a similar magnitude to those obtained upon activation of the vasodilator nerves or by inhibition of the vasoconstrictor nervous tone Thus a more detailed and systematic investigation is required before definite conclusions may be drawn about the effects exerted by acetylcholine on the oxygen uptake in skeletal muscle

Stimulation of hypothalamic vasodilator pathways produces vasoconstriction in the cutaneous and splanchnic vessels (Eliasson Lindgren and Uvnas 1952) If this vasoconstriction is pronounced it will result in an increased arterial pressure This pressure rise might via baroreceptor activation lead to inhibition of vasoconstrictor nervous tone in vessels of skeletal muscle (*cf* Bolme Ngai and Rosell 1967) Thus concomitant inhibition of vasoconstrictor nervous tone could explain why in the present study, a sustained increase in oxygen uptake was seen in some of the dogs on hypothalamic vasodilator nerve activation

Inhibition of the vasoconstrictor tone by sinus nerve stimulation led to a sustained increase in oxygen uptake provided that a concomitant increase in blood flow was produced This finding is similar to that seen in cats by Rosell and Uvnas (1962) An increased oxygen uptake has also been reported in skeletal muscle during the compensatory hyperemia obtained after cessation of vasoconstrictor nerve stimulation

(Pappenheimer 1941, Bolme and Gagnon to be published) As pointed out by Krogh (1929) only a small number of the capillaries in a resting muscle are simultaneously open It is probable that the vasoconstrictor tone usually present during rest plays a role in keeping the majority of the capillaries closed When the vasoconstrictor tone is inhibited, more capillaries will open up as a result of a loss of tone both in the arterioles and in the precapillary sphincters and a larger capillary surface area will be available for oxygen exchange (Renkin and Rosell 1962 b Cobbold *et al* 1963)

In contrast, Stainsby and Otis (1964) observed no consistent change in the oxygen uptake of the gastrocnemius plantaris muscles in the dog on decreasing the neurogenic vasoconstrictor tone although they obtained pronounced but transient blood flow increases One explanation for the discrepancy between our results and those of Stainsby and Otis (1964) might be that they measured the oxygen uptake by taking intermittent arterial and venous samples Stainsby and Otis (1964) might then have failed to observe a transient increase in oxygen uptake since this was not continuously followed

It is possible to link the observations made in this study to a previous investigation (Bolme and Novotny 1969) showing that in conscious dogs the sympathetic vasodilator nerves are activated as a conditional reflex in anticipation of exercise Thus *it appears that the vasodilator nerves might play a role in preparation for muscular* exercise by directing more blood to the muscles However, the oxygen uptake in skeletal muscles is probably regulated mainly by other mechanisms *e g* locally produced metabolites

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Subcellular Distribution of ^{3}H-noradrenaline in Adrenergic Nerves of Mouse Atrium — Effect of Reserpine, Monoamine Oxidase and Tyrosine Hydroxylase Inhibition

By

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Abstract

JONSSON, G and CH SACHS *Subcellular distribution of ^{3}H-noradrenaline in adrenergic nerves of mouse atrium—effect of reserpine, monoamine oxidase and tyrosine hydroxylase inhibition* Acta physiol. scand 1969 77 344—357

[illegible] n adrenergic nerves [illegible] or the methylester [illegible] ence histochemical [illegible] cause a pronounced decrease in the endogenous noradrenaline stores The subcellular distribution was practically identical in untreated, nialamide pretreated and H44/68 pretreated animals, approximately 30 % ^{3}H noradrenaline was recovered in the particulate fraction which in all probability contains the amine storage granules In the reserpine nialamide pretreated mice, however, most of the [illegible] and the granular uptake mechanism seem to operate The morphological studies disclosed differences in the adrenergic nerves when the noradrenaline taken up is mainly granularly or extragranularly stored since the varicosities are in the former case distinct and the latter case less distinct while the pre terminals are more prominent Methodological studies of the homogenization procedure for subcellular distribution studies have also been performed, the results of which are discussed in view of the experimental data obtained

It is now well established based on both biochemical and electronmicroscopic dence, that the peripheral adrenergic transmitter NA[1] is mainly stored in special neuronal storage granules (Euler and Hillarp 1956 Schumann 1958, Campos Shideman 1962 Potter and Axelrod 1962 Lundborg 1967, Hokfelt 1969) Exnously administered NA is rapidly taken up by the axonal membrane of the adrenergic neuron and subsequently incorporated into the storage granules, mainly by means of an ATP-Mg -dependent uptake mechanism This latter process can be efficiently blocked by reserpine Carlsson *et al* 1963, Euler and Lishajko 1963

[1] Abbreviations used NA = noradrenaline NM = normetanephrine MAO = monoamine oxidase

Carlsson 1966, Stitzel and Lundborg 1967, Lundborg 1967). However, a small part of the NA taken up intraneuronally can enter the granules via a reserpine resistant uptake mechanism (Stitzel and Lundborg 1967).

The uptake and accumulation of ^3H NA in adrenergic nerves of rat iris and mouse atria after reserpine pretreatment, MAO or tyrosine hydroxylase inhibition have been characterized in previous investigations (Jonsson *et al.* 1969, Sachs 1969). It was therefore considered of interest to study the subcellular distribution of exogenously administered ^3H NA in adrenergic nerves under the experimental conditions mentioned in order to be able to correlate histochemical, isotope and subcellular distribution data. Since the homogenization procedure is considered to be most critical in subcellular distribution studies (see Euler 1966) some methodological studies of this step have been performed.

Material and Methods

Methodological studies of the homogenization procedure

A. Subcellular distribution of ^3H NA

Mice (male N.M.R.I., 25 g) were given an i.v. injection of 20 µC ^3H NA (7—10 C/mmole) 30 min before ...

during homogenization a few atria from untreated animals were homogenized at 0° C after the addition of 0.1 µC ^3H NA. Radioactivity was extracted from the pellets and from the high speed supernatant with *n*-butanol/0.1 % HCl using 3 ml for the pellets and 4 ml for the extraction of 1 ml supernatant. An aliquot of 2 ml was measured in 10 ml toluene-ethanol (7 : 3) scintillation solution using a liquid scintillation spectrometer (Packard Model 3002). Recovery of the extraction procedure was found to be about 90 %. No corrections for recovery were made. Quenching was monitored by the addition of a standard amount of ^3H toluene.

B. Fluorescence histochemistry

The experiments performed were the same in principle as in A. except that the i.v. injection of ^3H NA was omitted and atria from 4 animals were used to get enough material. All of the various pellets (coarse, crude mitochondrial and microsomal fraction) were taken out of the centrifuge tube and frozen in liquid propane cooled with liquid nitrogen and then freeze dried. After this ...
for the histoche...
paraffin sections ...
microscope (for ...
tion in the fluor...
0.03 % $NaBH_4$ in 95 % isopropanol to check the specificity of the monoamine fluorescence (see Corrodi *et al.* 1964). The sections were further investigated in a fluorescence microspectrograph to obtain emission spectra and fluorescence intensity measurements (see Jonsson 1967).

Effect of reserpine, nialamide and H44/68 on the subcellular distribution of ^3H NA

A. Differential centrifugations

Four groups of mice were studied: 1) Untreated. 2) Pretreated with nialamide (100 mg/kg i.p., 2 hrs). 3) Reserpine (10 mg/kg i.p., 16 hrs) and nialamide (100 mg/kg i.p., 2 hrs). 4) H44/68, the methylester of α-methyl-*p*-tyrosine (500 mg/kg i.p., 16 hrs). The mice ...

various groups received an i v injection of 20 μC ^{3}H NA (7—10 C/mmole) 30 min before sacrifice The atria were dissected out washed and homogenized for 60 sec as described above The homogenates were centrifuged 2,000×g for 10 min to obtain the coarse fraction (P_1) its supernatant was centrifuged 107 000×g for 60 min to obtain a particulate fraction (P_2) and a high speed supernatant (S) The crude mitochondrial and microsomal fractions were thus centrifuged together The radioactivity was extracted from the pellets and supernatants as described above In some experiments atria were incubated for 30 min at +37° C in vitro

taken up

In 2 expts the pellets and supernatants were extracted with cool 0 4 N perchloric acid and the extracts chromatographed on Dowex 50 W X4 ion exchange columns (diameter 4 0 mm and height at pH 1, 120 mm) ^{3}H NA and ^{3}H NM were determined according to Carlsson and Waldeck (1963) and the acid and neutral catabolites estimated by shaking the effluent after acidification (pH 2) with 300 ml ether The volume was reduced under vacuum and radioactivity determined (see Rutledge and Jonasson 1967) No corrections for recovery were made

B Gradient centrifugations

The atria of 4 animals from each group were homogenized for 60 sec in 0 5 ml 0 25 M sucrose in 0 005 M phosphate buffer pH 7 4 containing 0 001 M $MgCl_2$ The animals had received 20 μC ^{3}H NA i v 30 min before sacrifice The homogenates were centrifuged 2 000×g for 10 min and 0 4 ml of the supernatant was carefully layered over a continuous sucrose gradient ranging from 0 25 M to 0 55 M sucrose (prepared according to Britten and Roberts 1960) The gradients were then centrifuged in a 3×5 ml swing out rotor 136 000×g (average force) for 60 min The centrifuge tubes were punctured at the bottom and fractions collected (7 drops/fraction) These were counted in 10 ml Bray scintillation solution with 4 ml ethanol (Potter and Axelrod 1963)

C NA assay and fluorescence histochemistry

Endogenous NA of whole mouse heart was determined after the various pretreatments according

pre

his

fors 1965 Sachs 1969) To allow adequate stretching of the tissue the atria had to be immersed in cool Krebs Ringer bicarbonate buffer for 10 min before whole mount preparation

Results

Methodological studies of the homogenization procedure

The ^{3}H NA recovered in the microsomal fraction when the time of homogenization was varied proved to be fairly constant for all the times used (see Table I) For the rest of the experiments a homogenization time of 60 sec (24 strokes) was chosen No redistribution of ^{3}H NA from the supernatant to cellular particles occurred during homogenization since almost all radioactivity (95 %) added was found in the supernatant

Fluorescence histochemistry of the pellets showed that in the coarse fraction parts of non homogenized adrenergic nerves could be identified (see Fig 1A) The number of nerves however was markedly reduced as compared with the non homogenized atria The adrenergic nerves innervating the vessels were the most resistant to the homogenization procedure No nerves could be observed in the crude mitochondrial and microsomal fraction but in the former fraction a very few small fluorescent dots were observed which might possibly have been non disrupted varicosities (see Fig 1B) The microsomal fraction simply showed an even fairly intense fluorescence (emission max 480 mμ) (see Fig 1C), which could be partly reduced by $NaBH_4$ treatment and restituted by renewed formaldehyde gas treatment

TABLE I Subcellular distribution of ^{3}H NA in mouse atria after various number of strokes during the homogenization procedure Distribution expressed in per cent For details see material and methods Each value is the mean of 4 determinations

Number of strokes	Coarse fraction	Crude mitochondrial + microsomal fraction	Supernatant fraction
12	44	16 (4 5+11 5)	40
24	33	21 (5+16)	46
36	35	21 (5 + 16)	44
72	34	22 (5 5+16 5)	44

Fluorescence histochemistry of mouse atria

Whole mounts of mouse atria were used to study the adrenergic nerves and nerve terminals in their full extent (*cf* Malmfors 1965) In the untreated mouse, the adrenergic nerves were arranged in a ground plexus located in the endocardium and having a fairly uneven distribution (Fig 2) This plexus consisted of characteristic varicose nerves several running together in bundles, which gave off single terminals with strongly fluorescent varicosities This type of preparation however, does not permit any conclusion as to whether the ground plexus extends into the myocardium In a few of the atria strongly fluorescent cells could be observed from which processes seemed to originate (see Fig 3) The registration of emission spectra showed the peak of emission to be 480 mμ, which is typical of formaldehyde induced catecholamine fluorescence (Fig 4) The fluorescence of these cells could also be abolished by $NaBH_4$ treatment, and restituted by renewed formaldehyde gas treatment

Atria from animals pretreated with nialamide showed the same innervation picture as those of untreated animals No difference in distribution or intensity could be observed

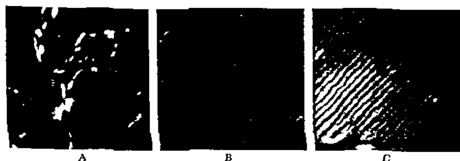


Fig 1 Histochemical demonstration of catecholamines in pellets of homogenized mouse atria A The coarse fraction (2 000 × *g* 10 min) shows strongly fluorescent parts of *adrenergic* nerves th[illegible]osities × 150
[illegible] an even weak fluorescence [illegible] (→) which may be non [illegible]
C The microsomal fraction (107 000 × *g* 60 min shows an even fairly intense fluorescence without any parts of nerves The striated appearance is an artefact due to difficulty in cutting this fragile pellet × 150

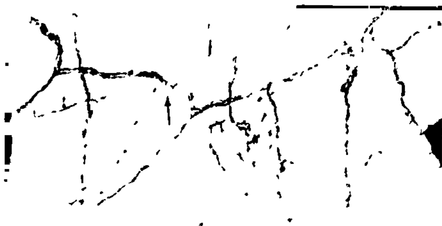


Fig 2 Histochemical demonstration of adrenergic nerves in a stretch preparation of atrium from untreated mouse The adrenergic nerves form a ground plexus in the endocardium of the atrium Four strongly fluorescent varicose nerves are seen running together (→) Single varicose terminals leave this bundle ×200

Reserpine caused a total disappearance of fluorescent nerves (Fig 5A) but a subsequent iv injection of 0 2 mg/kg dl-NA (30 min before sacrifice) resulted in a restitution of fluorescent nerves provided that MAO was inhibited (nialamide)



A B

Fig 3 A and B Strongly fluorescent clusters of cells in stretch preparations of mouse atrium Processes seem to originate from some of the cells ×200

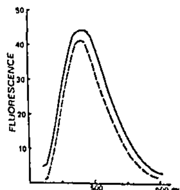
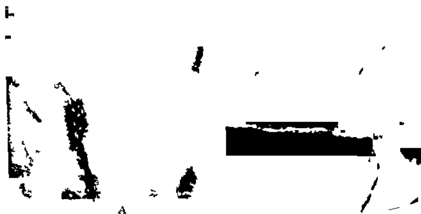


Fig 4 Emission spectra of a fluorescent cell (—) of mouse atrium and of a model protein layer prepared from a 2 % (w/v) aqueous solution containing 10^{-4} M dl NA (– – –) and exposed to formaldehyde gas at +80° for 1 hr Fluorescence expressed in arbitrary units

(Fig 5B) These nerves showed, however, a different morphology than those in the atria of untreated animals The varicosities were less distinct, and the terminals showed a reduced fluorescence intensity and smoother appearance Strongly fluorescent non terminals could also be seen

Pretreatment with the potent tyrosine hydroxylase inhibitor, H44/68 resulted in a marked reduction of the number of adrenergic nerve terminals, and of the fluorescence intensity (Fig 6A) A certain degree of variation in the depletion was observed between animals The injection of dl NA (0 2 mg/kg i v 30 min before sacrifice) resulted in a morphology, in respect of both the frequency of nerves and the fluorescence intensity, that was practically identical to that of atria from untreated animals (Fig 6B)



A B

Fig 5A Stretch preparation of mouse atrium, after pretreatment with reserpine (10 mg kg i p 16 hrs) No adrenergic nerves are visible, only a few autofluorescent pigments
B Mouse atrium after pretreatment with reserpine (10 mg/kg i p 16 hrs ... mg/kg i p 2 hrs and NA (0 2 mg/kg i v 30 min) The ground plexus is ... strongly fluorescent nerve bundles The varicosities are not very distinct ... a smooth appearance ×150



Fig 6A. Mouse atrium after pretreatment with H44/68 (500 mg/kg i p 16 hrs) Only a few very faint adrenergic nerves can be seen (→) × 150

B Mouse atrium after pretreatment with H44/68 (500 mg/kg i p 16 hrs) and NA (0 2 mg/kg i v 30 min) The adrenergic ground plexus has the same general appearance as that of the untreated mouse Bundles of varicose nerves, from which varicose terminals leave, can be seen × 150

The dose of reserpine used lowered the NA content of the whole heart (0 09 μg NA/g) by about 85 % compared with the untreated (0 60 μg NA/g), as determined by chemical assay H44/68 caused a reduction of the NA content (0 18 μg NA/g) to about 30 % of the normal

Subcellular distribution of ³H-NA in mouse atria

The analysis of the metabolism of ³H-NA (both *in vivo* and *in vitro*) after the different pretreatments revealed the formation of small amounts of metabolites (see Table I) The radioactivity extracted will thus mainly represent ³H-NA, 80 % or more

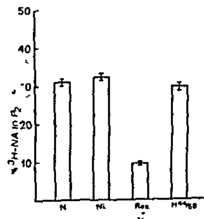


Fig 7 Uptake of ³H NA in the P_2 fraction of mouse atrium after various pretreatments For details see text The results are expressed as the amount of ³H NA in the P_2-fraction calculated as a percentage of that in P_2 + supernatant fractions The columns represent the mean ± S E M of 6—9 determinations. N = normal NL = nialamide Res = reserpine

TABLE II Uptake of ³H NA, ³H NM and ³H acid metabolites in subcellular fractions of mouse atria. The animals had received 20 μC ³H NA 30 min before sacrifice. Weight of 4 atria 15.7 mg (range 12.4 mg — 18.6 mg)

		³H NA		³H NM		³H acids	
		pg	%*	pg	%*	pg	%*
Untreated	P_1	156.8	86	18.2	10	7.3	4
	P_2	100.8	90	10.1	9	1.1	1
	S	217.4	91	16.7	7	4.8	2
		475.0	—	45.0	—	13.2	—
Nialamide	P_1	187.8	89	21.1	10	2.1	1
	P_2	134.9	90	15.0	10	—	0
	S	271.7	96	11.3	4	—	0
		594.4	—	47.4	—	2.1	—
Reserpine + nialamide	P_1	11.1	89	1.2	10	0.1	1
	P_2	8.4	88	1.1	12	—	0
	S	74.5	80	16.8	18	1.8	2
		94.0	—	19.1	—	1.9	—
H44/68	P_1	116.1	92	8.8	7	1.3	1
	P_2	122.8	96	2.6	2	2.6	2
	S	285.8	88	32.5	10	6.5	2
		524.7	—	43.9	—	10.4	—

* The figures give the percentage of the sum of the ³H activity found in respective fraction

After an i.v. injection of 20 μC (= 0.020 mg/kg) ³H NA the uptake and retention of ³H NA in the particulate fraction (P_2) was about 32 % of that found in both P_2 and the high speed supernatant (S) (Fig. 7). Nialamide pretreatment did not alter this distribution but the total amount of ³H NA extracted from the various fractions was somewhat increased (see Table II). Reserpine + nialamide pretreatment however altered the distribution markedly and only about 10 % was recovered in the P fraction. The total uptake and retention of ³H NA was also reduced to about 20 % compared to the untreated animal. H44/68 pretreatment resulted in a somewhat increased total uptake of ³H NA but the subcellular distribution was the same as that of the untreated animals. Practically the same distribution results were obtained after an i.v. injection of 0.2 mg/kg (10 mC/mmole) ³H NA 30 min before sacrifice. The subcellular distribution of ³H NA after *in vitro* incuba was essentially the same although the high speed supernatant contained more than in the *in vivo* studies

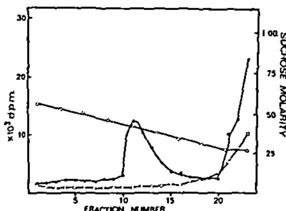


Fig 8 Subcellular distribution of ³H NA in mouse atrium homogenate The mice had received 20 μC ³H NA i v 30 min before sacrifice Homogenates were centrifuged in a 0 55—0 25 M sucrose gradient prepared as described in material and methods ●——● Untreated □——□ Pretreatment with reserpine and nialamide ○——○ sucrose molarity

The distribution of radioactivity in a continuous sucrose gradient (0 25—0 55 M) after centrifugation was practically identical in the untreated, nialamide and H44/48 pretreated animals The main part of the radioactivity was found at the top of the tube, corresponding to the supernatant and a small peak was found at the level corresponding to about 0 4 M sucrose (see Fig 8) In the reserpine + nialamide case however almost all of the radioactivity was located at the top of the gradient The general experience was that it was often difficult to obtain a clear cut resolution of the peak equilibrating at 0 4 M sucrose (*cf* Michaelson *et al* 1968) About 5 % of radioactivity layered on the gradient could be recovered from a very small pellet obtained at the bottom of the centrifuge tube

Discussion

In the last few years there has been a considerable interest in uptake mechanisms of the transmitter in the adrenergic neurons since these processes are considered to be of the utmost importance for the function of the nerves and they can also be modified by certain drugs The generally accepted view to day is that the adrenergic neuron possesses two uptake mechanisms, this view is based on experimental evidence of histochemical electron microscopical and biochemical origin (see among others Malmfors 1965 Carlsson 1966 Folkow *et al* 1967 Hamberger 1967, Iversen 1967 Hokfelt 1968 1969) One uptake mechanism the so-called membrane pump operates at the level of the axonal membrane and the other intraneuronally at the level of the amine storage granules Exogenously administered NA can thus be taken up by the membrane pump and then incorporated into the amine storage granules mainly via a Mg ATP dependent mechanism which is very sensitive to reserpine both *in vivo* and *in vitro* (Carlsson *et al* 1963 Euler and Lishajko 1963 Stjarne 1964 Iversen *et al* 1965 Lundborg 1967) Small amounts of transmitter can also be incorporated into the granules via a reserpine resistant mechanism (Stitzel and Lundborg 1967) The membrane pump is considered to play an important role for the uptake of circulating catecholamines and its physiological significance is to terminate the action of the transmitter released at the adrenergic nerve terminals whereas the granular uptake mechanism is of great importance for the total uptake

storage and retention of the NA taken up intraneuronally (see Iversen 1967)

The present work was initiated by the findings of Malmfors and Sachs (1968) that the morphology of restituted adrenergic nerves in rat irides differed after the i.v. injection of NA in rats pretreated with reserpine + nialamide and rats pretreated with the synthesis inhibitor H44/68 It was suggested from the morphological picture after i.v. administration of NA that in the reserpine + nialamide case, the amine taken up intraneuronally was mainly extragranularly located and in the H44/68 case probably granularly located In order to prove this more directly, a parallel histochemical and subcellular distribution study was made of NA taken up and retained in the adrenergic nerves of mouse atria.

The methodological studies of the homogenization procedure disclosed that homogenization of the atria was not complete even after 180 sec, since fluorescence histochemistry of the coarse fraction showed this to contain parts of non-disrupted adrenergic nerve terminals (see Fig 1A) This finding could very well explain the amount of ^{3}H NA recovered in this fraction (*cf* Euler and Lishajko 1965) Recovery of ^{3}H NA in the particulate fractions could not be increased by a longer homogenization time A homogenization time of 60 sec was therefore chosen for the rest of the fractionation studies Most of the NA taken up by the atria is most probably localized in the adrenergic neurons since immunosympathectomized atria which histochemically showed no adrenergic innervation have been found to take up only about 10 % ^{3}H NA *in vitro* (10^{-7} M 30 min) compared with atria from untreated animals (Sachs 1969) When atria were homogenized at 0° C after adding ^{3}H-NA to the homogenization medium almost 100 % of the radioactivity was recovered in the high speed supernatant, which shows that no redistribution of ^{3}H-NA occurs from the supernatant to cellular particles during homogenization at 0° C There are, however, several data to support the view that *in vivo* most of the NA is stored in the granules which indicates that there is a release of the bound amines during homogenization of the tissue (se Carlsson 1966, Euler 1966) No fluorescent nerve terminals could be observed in the crude mitochondrial or microsomal fraction but in the former fraction a few small fluorescent structures which might be non-homogenized varicosities could be observed In the microsomal fraction only an [illegible] and evenly distributed fluorescence could be seen to judge from the spectral and N [illegible] reduction results This is probably due partly to diffusely distributed NA The fact that no intact nerve terminals and only very few—if any—varicosities were found in the crude mitochondrial fraction indicates that most of the amine taken up in these fractions represents a granular uptake In the studies after various pretreatments crude mitochondrial and microsomal fractions were therefore centrifuged together although this fraction must be considered as fairly impure, since it is contaminated with mitochondria other cell organelles and fragments The mitochondria, however have been shown not to take up any NA (Potter and Axelrod 19[illegible])

Fluorescence histochemistry showed the endocardium of the atria to [illegible] characteristic adrenergic ground plexus and blood vessel innervation (*cf* [illegible] *et al* 1963 Ehinger *et al* 1966 Lipp and Rodin 1968) From this it

decided whether or not the ground plexus extends into the myocardium. The fluorescent cells sporadically found in the atria doubtless contain a catecholamine, and are probably of the same type as described by Jacobowitz (1967, cf. Boyd 1960 and Bloom et al. 1961). Since they are relatively seldom seen and are probably of a 'chromaffin' cell type, the possible uptake of NA in these cells must be very small or negligible.

Reserpine caused a complete disappearance of the fluorescence of the adrenergic nerves, and the NA content of the whole heart was reduced to 15 % of the normal value (see Carlsson 1965). The fluorescence of the adrenergic nerves could be restituted by an i.v. injection of dl-NA (0.2 mg/kg 30 min before sacrifice), but the animals had to be pretreated with nialamide in order to prevent the intraneuronal catabolism of NA taken up (cf. Malmfors 1965). The appearance of the nerves had changed, however, since the varicosities were less distinct and the whole terminal had a smoother appearance. The pre-terminal axons exhibited a very strong fluorescence intensity (cf. Malmfors 1965, Malmfors and Sachs 1968). In this case too the subcellular distribution differed from the normal, since most of the ^3H-NA was found in the high speed supernatant, supporting the view that reserpine has a blocking effect on the granular uptake mechanism. These findings are consistent with the results of Stitzel and Lundborg 1967 (see also Carlsson et al. 1963, Euler and Lishajko 1963, Iversen et al. 1965). A small uptake had, however, occurred in the particulate fraction (P_2) which was reserpine-resistant. This may be due to the granules possessing two uptake mechanisms, one Mg^{++}-ATP-dependent which can be blocked by reserpine and another which is not affected by this drug (see Stitzel and Lundborg 1967). It has been shown, however, that the reserpine resistant uptake is dose dependent, since Stjärne (1964) and Euler and Lishajko (1965), using a high medium concentration of NA, found in vitro that a reserpine resistant granular uptake was observed. Similar results have been obtained in fluorescence histochemical and electron microscopical studies on intact adrenergic nerves (Hamberger and Malmfors 1967, Hökfelt 1968). Part of the uptake in the P_2 fraction may also be due to this fraction containing a few varicosities or due to non-specific binding of NA to proteins. Tyrosine hydroxylase inhibition produced by H44/68 resulted in a marked reduction of the fluorescence in the adrenergic nerves, and chemical assay showed that the total NA content of the whole heart was reduced to about 30 % of normal. Since H44/68 efficiently inhibits the rate limiting step (tyrosine-dihydroxyphenylalanine) in the NA biosynthesis, a depletion of the endogenous NA store is produced (Spector et al. 1965, Andén et al. 1966, Corrodi and Malmfors 1966, Udenfriend 1966). After an i.v. injection of NA the nerve fluorescence was restituted and the characteristic picture of varicose nerves was seen. It was unnecessary to inhibit MAO, contrary to the situation when the animals had been pretreated with reserpine. H44/68 is itself no MAO inhibitor (Jonsson et al. 1969). The subcellular distribution after H44/68 showed the same pattern as that from untreated animals, implying that the granular Mg-ATP uptake mechanism was intact although the granules must initially have been almost empty. The total uptake

and retention of ^{3}H NA in the H44/68 pretreated animals was only slightly higher than in the untreated (*cf* Laverty and Robertson 1967) and with the doses of NA used the amount taken up could not account for a complete refilling of the almost emptied stores, not even after a high dose (0 2 mg/kg) Similar results have been obtained in *in vitro* studies on rat iris and mouse atria (Jonsson *et al* 1969 Sachs 1969) It may be suggested that exogenous NA does not easily mix with the original NA pool or pools Recently electronmicroscopical data speaking in favour of this view have been published (Hökfelt 1968) It has been found, however, that rat heart NA levels reach approximately normal values after perfusion for 10 minutes with a medium containing 10^{-7}—10^{-6} M NA in animals previously depleted of their endogenous NA with decaborane (Bhattacharya 1968)

MAO inhibition alone did not affect the morphology or the subcellular distribution of ^{3}H NA of the adrenergic nerves although the total uptake was increased

Most of the NA in the particulate fraction equilibrates with a concentration of about 0 4 M sucrose, when a continuous gradient is used ranging from 0 25 to 0 55 M a result which is very close to that of Michaelson *et al* (1968) Some (25 %) of the radioactivity was, however, associated with heavier particles Whether these are another type of storage granules and/or varicosities, or a co-precipitation of granules equilibrating at 0 4 M sucrose with other cell fragments is a matter that must await further investigations (*cf* Roth *et al* 1968)

From the results presented in this paper it has been confirmed that reserpine acts as a potent inhibitor of the NA uptake in the amine storage granules although there is a small reserpine resistant uptake After tyrosine hydroxylase inhibition by H44/68 there is a pronounced depletion of the endogenous NA stores in the mouse atrium but in contrast to the reserpine-case the subcellular distribution of exogenously administered ^{3}H NA is principally the same as in untreated animals Both the membrane pump' and the granular uptake mechanisms are thus intact after tyrosine hydroxylase inhibition but the uptake and retention of ^{3}H NA is not as great as could be expected to account for a complete refilling of the emptied stores The morphology of the nerves exhibits a different appearance when the NA taken up is mainly granularly or extragranularly stored since the varicosities are in the former case distinct and in the latter less distinct at the same time as the preterminals are more prominent

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Effects of Slow Infusions of KCl into the 3rd Brain Ventricle

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Abstract

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During slow (7 5 µl/min) infusions of strongly hypertonic (0 4—0 85 M) KCl solutions into the anterior part of the 3rd brain ventricle of the goat, a series of behavioural reactions de-

It is concluded that a rather selective non specific stimulation of diencephalic mechanisms may be obtained by slow infusions of KCl into the anterior part of the 3rd ventricle. The experiments have given no evidence that an elevated K^+ concentration in the diencephalic region stimulates renal K excretion specifically

Hypertonic NaCl and some other hypertonic solutions were previously infused into the 3rd brain ventricle of the goat for the purpose of studying central control of water and electrolyte balance (Andersson Olsson and Warner 1967 Andersson Dallman and Olsson 1969 a) Intraventricular infusions of hypertonic KCl were omitted in these studies because preliminary infusions had been found to cause certain striking effects which apparently were due to a non specific stimulation of diencephalic neurons during their exposure to an abnormally high K concentration. This observation suggested that K ion stimulation via the 3rd ventricle might provide some new information on diencephalic mechanisms. Therefore, a series of experiments have been undertaken in which the reactions of goats to slow infusions of KCl solutions into the 3rd ventricle have been studied

Methods

Animals The experiments were performed in 9 adult female goats (b w 30—40 kg. The animals were used for experiments over periods from 2 to 5 months. The same goats were also subjected to experiments in which the effect of intraventricular infusions of hypertonic NaCl on renal Na^+ excretion and on water intake was studied (Andersson *et al* 1969 a). The minimum interval between experiments in each single animal was 3 days. The goats were routinely

confined in metabolism cages by means of collars and all experiments were conducted in these cages. The animals had access to chaffed hay and water *ad lib*, except for certain periods of time during the experiments when water had to be withheld in order to prevent fatal overhydration. Each afternoon the goats were given 400 g of commercial grain mix and 6 g of NaCl either added to the grain mix or dissolved in some warm water. Room temperature was maintained at 20±1° C.

Brain implantations and infusion technique. All animals were prepared with permanent cannulae in the anterior part of the 3rd brain ventricle. To minimize the "dead space" the infusions were performed via an inner cannula which was filled with the KCl solution and was inserted to the ventricular end of the permanent cannula before the infusion was started. The operation and infusion techniques have been described in detail earlier (Andersson *et al.* 1967). A total of 35 experiments involving intraventricular infusions of KCl were made. The rate of infusion was always maintained at 7.5 µl/min and the duration of the infusion periods varied between 3 and 60 min. KCl concentrations from 0.15 to 0.85 M were used.

Hydration. In some experiments a water diuresis was established by giving the goats 100 ml of tepid water/kg b.w. by stomach tube 2 hrs before the intraventricular infusion was started.

Samples. Blood samples were obtained in heparinized syringes from the jugular vein. Urine samples were taken at 10 or 15 min intervals via a retention catheter inserted in the urinary bladder. During one experiment involving determinations of renal inulin clearance a priming dose of inulin was followed by infusion of inulin (12 ml/hr) into the jugular vein in amounts which produced a maintained plasma inulin concentration between 8 and 12 mg/100 ml. At the end of each clearance period (15 min) the urinary bladder was washed two times with 10 ml of water.

Analyses. Plasma and urine Na^+ and K^+ concentrations were determined using an EEL flame photometer and the Cl^- concentration of the fluids was determined by the method of Brun (1949). For determinations of urine osmolarity a "Knauer" osmometer was used. Inulin concentrations in plasma and urine were determined by the Josephson and Godin modification (1943) of the diphenylamine reaction.

Results

The infusions of KCl into the 3rd ventricle were generally started around 11 a.m. At this time of the day the goats had eaten to satisfaction from the morning refill of the hay bin. They had also performed postprandial drinking and were thus no longer interested in hay and water available in front of them.

General characteristics of the response to the infusion

Although certain individual differences were seen, the behaviour of the goats during infusions of KCl solution into the anterior part of the 3rd ventricle followed a rather regular pattern. During prolonged (30—60 min) infusions of the stronger solutions (0.4—0.85 M) a series of reactions developed in a typical time sequence. An urge to drink which rapidly grew very strong, became apparent after about 10 min of infusion. At the same time the respiratory rate started to increase from about 20/min to levels between 100 and 200/min. While retaining the urge to drink and the polypnea, 5 of the 9 goats started to eat another 10 to 20 min later. In 3 animals this feeding response attained the character of ravenous hunger. Excited snorting and other signs of minor rage accompanied the fully developed hyperphagia. These behavioural effects outlasted the infusion period by about half an hour.

Thirst and polypnea also developed during prolonged infusions of the weaker (0.15—0.3 M) KCl solutions although there was a longer latency time. Obvious hyperphagia was, however, not observed under these conditions.

A constant effect of the infusions was increased renal Na^+ excretion. Brief (3—5 min) infusions performed in 3 of the goats during hydration also invariably caused a

temporary inhibition of their water diuresis The following is a more detailed description of the effects of the intraventricular KCl infusions on the water and electrolyte balance of the goats

Thirst

Since intraventricular infusions of NaCl solutions also were made in the present goats, it was possible to compare in the same animal the thirst response to infusions of equimolar NaCl and KCl into the anterior part of the 3rd ventricle NaCl solutions which were approximately isotonic (0 15 M) to the cerebrospinal fluid (CFS) did not cause drinking However, even 0 15 M KCl induced an urge to drink during prolonged infusion Comparisons made between the effects of equimolar, hypertonic (0 3—0 85 M) solutions of NaCl and KCl revealed other characteristic differences In the pre hydrated animal infusions of hypertonic NaCl did not induce drinking whereas infusions of equimolar KCl elicited a strong urge to drink, whether the goats had been hydrated or not In the non hydrated goat prolonged infusions of hypertonic NaCl induced the cumulative and controlled drinking described earlier (Andersson *et al* 1967) In contrast, the drinking caused by equimolar KCl was of the same excessive character as that obtained by electrical stimulation in the anterior medial part of the hypothalamus of this species (Andersson and McCann 1955) The thirst was not quenched by continuous drinking and the water bucket had to be removed in order to prevent serious over hydration of the goats In spite of the more excessive and uncontrolled drinking the latency time before thirst appeared was always longer during KCl infusion than during infusions of equimolar strongly hypertonic (0 3—0 85 M) NaCl solutions The differences between K^+ and Na^+ induced drinking are illustrated in Fig 1

Natriuresis

It has earlier been shown that slow infusions of hypertonic NaCl solution into the 3rd ventricle of goats maintained on a salt supplemented diet provokes natriuresis reaching maximum about one hr after the onset of the infusion Some increase in

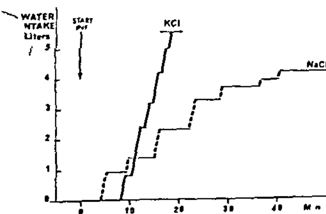


Fig 1 Excessive, and almost continuous drinking elicited during a slow (7 5 µl/min) infusion of 0 85 M KCl into the anterior part of the 3rd ventricle For comparison is shown the more controlled cumulative drinking seen during a similar infusion of equimolar NaCl in the same goat The KCl induced drinking had to be interrupted (*horizontal arrows*) to prevent serious over hydration The intraventricular infusions were started at zero time Note the longer latency time for the KCl induced drinking

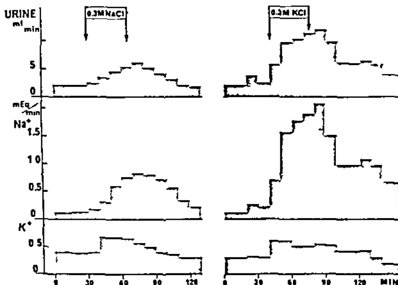


Fig 2 The natriuretic effect of a 30 min infusion (7.5 µl/min) of 0.3 M NaCl into the anterior part of the 3rd ventricle (*left*), compared to the much more pronounced natriuretic response to a similar infusion of equimolar KCl in the same goat (*right*). The increase in renal K^+ excretion remained of the same order in both experiments

renal K^+ excretion also occurs. Determinations of inulin clearance have indicated that the glomerular filtration rate (GFR) increases and that the relative tubular reabsorption of Na^+ decreases to some extent during this natriuresis (Andersson *et al* 1969 a)

The intraventricular infusions of KCl invariably provoked a similar natriuretic response and a moderate increase in renal K excretion which was comparable to that seen during natriuresis induced by the intraventricular infusion of hypertonic NaCl (Fig 2). Studies of inulin clearance performed in one of the KCl experiments indicated a 20 % increase in GFR during peak natriuresis. However comparisons made between the effectiveness of equimolar NaCl and KCl in provoking natriuresis revealed differences. Prolonged (30 to 60 min) infusions of isotonic NaCl (0.15 M) did not cause any increase in renal sodium excretion while considerable natriuresis was obtained by the infusion of KCl solutions of the same low molarity. When the effects of equimolar hypertonic solutions of NaCl and KCl were compared in the same animal, the natriuretic response to intraventricular infusions of KCl was always considerably greater and more sustained than the response to NaCl infusions (Fig 2)

In pre hydrated goats the natriuretic effect of brief (5 to 10 min) intraventricular infusions of even strongly hypertonic (0.5—0.85 M) NaCl was weak or sometimes absent. Corresponding infusions of KCl however effectively elicited natriuresis also in pre-hydrated goats. Due to this fact serious hyponatremia happened to be induced in one of the goats. With a 1 hr interval two 10 min infusions of 0.85 M K

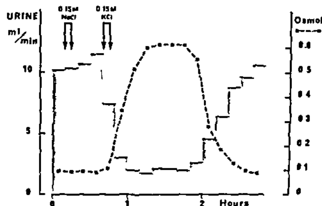


Fig 3 Temporary inhibition of the water diuresis and rise in urine osmolarity caused by a brief (5 min) infusion (7.5 µl/min) of isotonic (0.15 M) KCl into the anterior part of the 3rd ventricle of a hydrated goat. Note the lack of response to a similar infusion of isotonic NaCl

made into the 3rd ventricle of the pre hydrated animal. By inadvertence water was available after the KCl infusion and the goat made thirsty by the second infusion emptied the 5 l bucket in one drinking sequence. During the following 3 hrs the renal Na^+ and Cl^- excretion was of the order of 1.5 mEq/min resulting in a fall of plasma Na to 115 mEq/l and plasma Cl to 82 mEq/l

Inhibition of water diuresis

Brief infusions of hypertonic NaCl into the anterior part of the 3rd ventricle have been found to cause a temporary inhibition of the water diuresis of hydrated goats (Andersson *et al* 1967). The inhibition is apparently due to release of antidiuretic hormone (ADH) from the neurohypophysis since the effect disappears when experimental diabetes insipidus has been produced by interruption of the hypothalamo-neurohypophyseal connections (Andersson, Dallman and Olsson 1969 b)

Brief (3—5 min) intraventricular infusions of KCl solutions performed in 3 of the present goats during hydration resulted in a similar temporary inhibition of the water diuresis with a concomitant rise in urinary solute concentration. In contrast to NaCl however KCl effectively inhibited the water diuresis also when infused as practically isotonic (0.15 M) solution (Fig 3)

Discussion

A rise in the extracellular K concentration stimulates excitable cells by depolarisation (Fatt and Katz 1951). Consequently injections of potassium salts into the cisterna magna and into the lateral ventricles have been found to exert a non specific stimulation of the central nervous system (*cf* John, Tschirgi and Wenzel 1959)

In the present experiment KCl was slowly infused into the anterior part of the 3rd brain ventricle. From there it may reach large areas of the brain by diffusion within the CSF throughout the ventricular system. The transport of infused K ions from the 3rd ventricle via the mesencephalic canal to the 4th ventricle is also facilitated

by CSF flow in that direction at a rate of about 0.15 ml/min in the adult goat (Heisey, Held and Pappenheimer 1962). However during a constant intraventricular infusion of a solution with a K concentration considerably above that of the CSF the K ion density must remain highest near the cannula opening. In the goat the clearance of K^+ from CSF perfused through the ventricular system is four times greater than the clearance of Na^+ (Heisey *et al.* 1962). Using a similar technique, Cserr (1965) has shown that 80 % of K^+ in the perfusate leaves the ventricular system by ionic flux across the ependyma into the brain tissue. Therefore the parts of the brain which in the present experiments most likely have been exposed to the highest K concentration are the medial hypothalamus, the medial preoptic region and the anterior ventral massa intermedia. This is also evidenced by the behavioural effects observed during the infusions. The first obvious reactions of the animals were an urge to drink and polypnea. The same reactions can be induced in this species by electrical stimulation in the anterior medial hypothalamus (thirst) and medially in the preoptic region (polypnea) (Andersson and McCann 1955, Andersson, Grant and Larsson 1956). Less consistent and at a later stage during the intraventricular infusions of KCl hyperphagia developed. In the goat (Larsson 1954) and other species this effect is obtained by electrical stimulation in the lateral hypothalamus. It appears that the intraventricular infusions of KCl gradually increased the extracellular K in these parts of the brain to a level causing non-specific stimulation, and that areas adjacent to the ventricular wall were affected in the first hand.

Evidence has been produced that hypothalamic "osmoreceptors" control the release of ADH from the neurohypophysis (Verney 1947, Jewell and Verney 1957) and a similar hypothalamic mechanism may participate in the regulation of water intake (*cf.* Andersson 1966). That the KCl infusions stimulated both these mechanisms in a non-specific manner and not osmotically is indicated by the observations that brief infusions of isotonic KCl caused an inhibition of the water diuresis of hydrated goats, and that prolonged infusion of isotonic KCl induced thirst in both hydrated and not hydrated animals. It has been shown that a rise in the K concentration of the medium causes a release of ADH from incubated neurohypophyseal tissue (Thorn 1966). However only minute amounts of KCl needed to be infused into the 3rd ventricle to cause a marked inhibition of the water diuresis (Fig. 3). Therefore a direct effect of the infused K ions as far distant as in the posterior lobe of the pituitary seems unlikely. The possibility that ADH might have been released from the neurosecretory cells within the hypothalamus appears to be eliminated by the observation that such a release does not occur due to potassium depolarization *in vitro* (Bie and Thorn 1966).

The intraventricular infusions of strongly hypertonic NaCl (0.3—0.85 M) induced the urge to drink sooner than did similar infusions of equimolar KCl. The relatively long latency time before drinking occurred during the intraventricular KCl infusions indicates that the extracellular K concentration had to reach a certain level before excitatory depolarization of the "thirst" neurons took place. With a transependymal flux of K much faster than that of Na (Heisey *et al.* 1962), it appears that a much

smaller rise in the extracellular Na^+ concentration is needed to stimulate the "thirst" neurons. That the rise in Na^+ concentration may be a natural stimulus to the hypothalamic thirst mechanism is also indicated by the lack of other behavioural effects during the infusions of hypertonic NaCl. A further indication is that (in contrast to thirst induced by the intraventricular KCl infusions) the NaCl elicited thirst was temporarily quenched by the drinking of water (Fig. 1).

The natriuretic response to infusions of hypertonic NaCl into the 3rd ventricle of the goat suggests a central control of renal Na^+ excretion exerted from the diencephalic region in this species (Andersson *et al.* 1969 a). The much more pronounced natriuresis elicited by similar infusions of equimolar KCl (Fig. 2) may reflect a non-specific stimulation of such a mechanism. However, the present experiments gave no suggestive evidence that an elevated K^+ concentration in the diencephalic region stimulates renal K^+ excretion specifically.

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The Effect of Gastric Juice Extracts on Histamine as a Gastric Secretory Stimulant

By

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Abstract

SEMB, L S *The effect of gastric juice extracts on histamine as a gastric secretory stimulant* Acta physiol scand 1969 77 365—371

Extracts from the isolated, vagally denervated gastric antral and fundic pouches (Heidenhain) of dogs were prepared by dialyzing against water and lyophilization The extracts were incubated with histamine for 1/2 hr and then injected s c into dogs with Heidenhain pouches [illegible] and in one of the dogs receiving fundic juice extract there was an 18 % increase ($P<0.01$). It was concluded that gastric juice extracts seemed not to interfere with the stimulatory effect of histamine on the oxyntic glands

Gastric secretion of acid has been shown to be inhibited by the i v injection of extracts from gastric juice Extracts prepared from human anacid gastric juice and juice of isolated antral pouches in the dog are the richest source of this inhibitor (Brunschwig *et al* 1939, 1940, Hood, Code and Grindlay 1953 Semb 1966*a*, *b*) However, the mode of action of this "gastric inhibitory substance" (GIS) is not clear Previous studies have shown that intravenous injection of extracts from gastrointestinal secretions inhibit acid responses to histamine (Semb 1969) In preliminary tests, s c or i m administration of GIS did not affect the response to i v histamine The purpose of the present study was to test whether GIS could in some way alter the stimulating effect of histamine on the oxyntic glands If GIS acts by breaking down or otherwise changing histamine, this should be elicited by *in vitro* incubation and s c injection of the two If a change of the histamine was effected, this would give a reduced secretory response as compared to an injection of histamine alone

The following is a report of experiments where extracts from the antral and fundic juice of dogs were incubated with histamine and assayed by s.c. injection in Heidenhain pouch dogs.

Material and methods

Animals

5 mongrel dogs weighing between 7 and 15 kg were equipped with vagally denervated fundic pouches (*Heidenhain*) and two dogs with vagally denervated antral pouches. The technique for pouch construction is described earlier (DeVito and Harkins 1959, Rheault *et al.* 1965, Semb 1966*b*).

Extracts

The dialyzed and lyophilized extracts of the secretions from both the fundic and the antral pouches were prepared as detailed earlier (Semb 1969).

Experimental procedure

The dogs were brought to the Pavlov stand after an overnight fast (14—18 hrs). It was ascertained that there was no secretion from the Heidenhain pouch for a period of approximately 1 hr. The response to subcutaneous injection of 100 µg histamine diphosphate (corresponding to 36.4 µg base) in 5 ml 0.9 % NaCl was measured for 75 min. In one series of 31 expts. in 5 dogs, a second dose of histamine was given after a period of about 1/2 hr of secretory arrest. In another series of experiments, the first histamine injection was followed by a s.c. injection of a mixture of 100 µg histamine acid phosphate in 5 ml of 0.9 % NaCl to which was added 1 mg/kg b.w. of either antral (36 expts. in 5 dogs) or fundic extract (15 expts. in 3 dogs). The histamine and extracts were mixed together at room temperature for 1/2 hr in a beaker with continuous stirring before the s.c. injection.

Collection of gastric juice

The gastric juice was collected by gravity drainage into graded centrifuge tubes fitted to the cannula from the Heidenhain pouch by means of a plastic adapter.

Analysis of gastric juice

The gastric juice was collected for a total of five 15 min periods after the s.c. injections. The volume of each sample was measured directly in the graded centrifuge glass. In the second and third sample aliquots of 1 ml of gastric juice were titrated to pH 7.0 against 0.01N NaOH using neutral red as indicator. The average peak concentration was calculated as a mean of the determined values for these two periods in each dog. The peak acid output was also calculated in a similar way, multiplying volume by concentration for these two periods.

Rectal temperatures

Rectal temperatures were measured in each animal every 15 min during the tests by means of a USCO clinical thermometer. No changes were recorded in the rectal temperatures in any of the animals.

Statistical analysis

Means and standard error of the means (SE) and the significance of difference between means utilizing the Student's t test were calculated according to Bradford Hill (1955).

Results

The effect of histamine alone (Table I)

Gastric acid secretion started in the first 15-min period after the subcutaneous injection of histamine and reached maximal values in the second and third 15 min period. A gradual fall to secretory arrest was observed in the fourth and fifth 15 min periods (Fig. 1). Although there was a considerable variation in the response on different days, there was an acceptable reproducibility of volumes of gastric juice, the

TABLE I The response to repeated subcutaneous injections of histamine in Heidenhain pouch dogs (31 expts in 5 dogs)[1,2]

Dog	Number of expts	Volumes of gastric juice ml/75 min		Peak concentration meq/l		Peak acid output μeq/15 min	
		Histamine	Histamine	Hist	Hist	Hist	Hist
La	9	9.7±1.1	9.4±1.1	139±3.5	139±2.2	429±58	416±50
Si	7	10.9±1.0	11.0±0.3	124±3.7	124±3.7	483±47	450±34
Sa	5	9.0±1.2	11.5±1.3	113±3.2	127±4.2***	350±37	529±55**
Ba	5	11.5±0.3	10.3±0.4	102±8.2	106±4.2	419±62	351±52
Pe	5	9.6±0.5	10.1±0.6	86±8.1	95±7.8	299±46	365±38

[1] All results are given as mean±S.E.M.
[2] The results marked with asteriks indicate significance of difference from controls (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$)

concentrations and peak output of titratable acid in four of the five dogs. In the fifth dog (Sa) an increase in all three parameters was observed after the second histamine injection.

The effect of histamine combined with antral extract (Table II)

The response to injection of histamine combined with antral extract was not significantly different from that observed after histamine alone in 3 of the 5 dogs. In one dog (Si), there was a statistically significant decrease in both volume of gastric juice and concentration of titratable acid, while in another (Sa) the average volumes of gastric juice were unchanged, but an increased concentration and output of titratable acid was found.

The effect of histamine combined with fundic extract (Table III)

The response to subcutaneous injection of histamine incubated with fundic extracts was on the average not statistically significantly different from that obtained with the injection of histamine alone in 2 of the dogs. In the third dog (Ba) a statistically significant increase in gastric secretion was found.

Discussion

The double histamine test has been in use for experiments on gastric secretion since the 1920's (Lim 1924, Ivy and Javois 1925), and has been extensively used for the study of gastric secretory inhibition (Polland 1931, Rivers, Osterberg and Vanzant 1936, Gray, Wierczrowsky and Ivy 1940, Friedman and Sandweiss 1942, Janowitz and Hollander 1957). The use of two successive stimuli for demonstration of inhibition by injection of a test substance to possibly alter the response to the second injec-

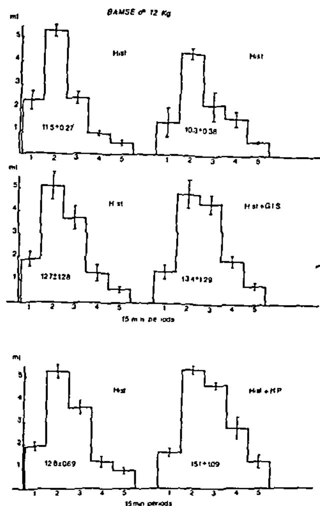


Fig 1 The effect of repeated subcutaneous injections of histamine (Hist, upper diagram), histamine and histamine incubated with Gastric inhibitory substance (Hist + GIS, middle diagram) and histamine and histamine incubated with Heidenhain pouch juice extract (Hist + H P) on gastric secretory volume response in a 12 kg dog Each diagram represents the average of 5 expts. The vertical bars indicate the standard error of the mean (SE) The numbers given in the diagrams represent the total volume for the 75 min period ± SE

TABLE II The response to subcutaneous injection of histamine combined with antral extract (GIS) in Heidenhain pouch dogs (36 expts in 5 dogs)[1 2]

Dog	Number of expts	Volumes of gastric juice ml/75 min		Peak acid concentration (meq/l)		Peak acid output (μeq/15 min)	
		Hist	Hist + GIS	Hist	Hist + GIS	Hist	Hist + GIS
La	11	7 8±1 1	8 6±1 5	140±3 4	138±2 4	418±75	422±89
Si	10	13 3±1 2	9 7±0 4***	127±3 4	119±2 7**	563±60	374±61**
Sa	5	11 3±2 6	11 7±2 0	110±6 2	127±3 4***	497±79	603±85***
Ba	5	12 7±1 3	13 4±1 3	119±2 7	112±4 4	462±47	524±47
Pe	5	9 1±1 3	11 2±1 5**	78 2±8 1	73±8 4	300±56	290±54

[1 2] See Table I

TABLE III The response to subcutaneous injections of histamine and histamine combined with fundic extract (HP) in Heidenhain pouch dogs (15 expts in 3 dogs)[1,2]

Dog	Number of expts	Volumes of gastric juice ml/75 min		Peak acid concentration (meq/l)		Peak acid output μeq/15 min	
		Hist	Hist +HP	Hist	Hist +HP	Hist	Hist +HP
Si	5	7 3±1 8	6 5±0 9	117±7 5	110±7 4	477±126	386±66
Sa	5	9 9±1 7	9 9±0 9	118±4 0	127±3 5	474±69	549±35
Ba	5	12 8±0 7	15 3±1 1**	129±3 9	123±4 6	562±33	603±27

[1,2] See Table I

tion of stimulant implies firstly, that one can predict the response of the second injection of stimulant in comparison with the first Secondly, the dose of stimulant or the stimulus must not be so great as to mask the possible inhibitory effect since an inverse relationship has been shown between the sensitivity of gastric secretion to inhibitors and the secretory rate (Gray, Bradley and Ivy 1937 Code *et al* 1949) Thirdly, it is of great importance that the timing of stimulus and inhibition is correct i e, that the inhibitor acts at the same time as the stimulus

Regarding the first implication of the method there was an appreciable variation in the day to-day response to a fixed dose of histamine injected subcutaneously in the present study, and also a variation in the response to the second injection of histamine compared to the first However when comparing the averages for five or more experiments in each dog there were no statistically significant differences between the mean responses in four of the dogs while a significant increase of 27 % in response to the second injection of histamine could be demonstrated in one dog (P<0 001) A tiring out of the gastric secretory cells to repeated injection of histamine does not seem to have occurred in these animals which agrees with the results found after continuous injection of the drug (Code *et al* 1949) It should therefore be possible to compare average responses from each dog in this study of extracts from either the antrum or the fundus incubated with a fixed dose of histamine with the response to histamine alone

The dose of histamine chosen in the present experiments was of the same magnitude as that giving a response of approximately 50 % of maximum on intravenous injection during 1 hr (Code *et al* 1949) The doses of the extracts from antrum or fundus were also of the same order of magnitude as those shown previously to give inhibition upon i v injection (Semb 1966*a* 1969) In earlier studies it was shown that inhibition of gastric acid secretion took place about 3/4—1 hr after the i v injection of the antral extracts In the present study the material was incubated with histamine for 1/2 hr before injection into the dog The maximal secretory response to subcutaneous histamine is reached after 1/2—3/4 hr The failure to demonstrate

any change in response to histamine incubated with gastric extract can therefore be taken as a failure of extracts to interfere with the stimulatory effect of histamine on the oxyntic glands

The injection of antral extracts together with the second dose of histamine showed no statistically significant different response as compared with the control in four of the five dogs In one dog however, the volume response was lowered on average 27 % ($P<0\ 01$) *Furthermore, the concentration and output of titratable acid* showed significant reduction Thus, in this one dog, the possibility occurred that the histamine was altered by the addition of antral extract However, the dog which showed this inhibition also showed the *greatest variations in day to day response* and the result is unreliable from this point of view In the experiments with fundic juice extracts there was no change in the response to the injection of incubated histamine as compared to histamine alone in two of the dogs while in the third there was an 18 % increase ($P<0\ 01$) in volume of gastric juice, but the average concentration and output of acid was not statistically significantly different from controls

The failure to show inhibition in the majority of tests in this study seems to favour *the concept that antral or fundic extracts under the conditions of these experiments* do not act by interfering with the stimulatory effect of histamine on oxyntic glands

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Studies on the Relaxing Actions Mediated by Stimulation of Adrenergic α- and β-Receptors in Taenia Coli of the Rabbit and Guinea Pig

By

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Abstract

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In experiments on taenia coli from rabbits, a study was made of the mechanisms whereby catecholamines induce a relaxing effect. In a low concentration ($\leq 2.1 \times 10^{-6}$ g/ml) l-phenylephrine selectively stimulated adrenergic α receptors; this effect was blocked completely by the adrenergic α blocking agent dibenamine. In a low concentration ($\leq 3.7 \times 10^{-8}$ g/ml) l-isoprenaline selectively stimulated β receptors; this effect was blocked completely by the adrenergic β receptor blocking agent sotalol. In higher concentrations, phenylephrine and isoprenaline stimulated both adrenergic α- and β receptors; their relaxing effect could only be blocked completely by a combination of dibenamine and sotalol. In taenia coli from rabbits the threshold concentration for β receptor mediated relaxation was lower than that for relaxation induced via α receptors; in taenia coli from guinea pigs the reverse was found. The relaxant effect induced via adrenergic α receptors was blocked selectively by factors such as cold treatment, digitalis glycosides, desoxycorticosterone, which could be assumed to decrease the ionic gradients of K and Na across the cell membrane or reduce its ionic permeability. — On successive reduction of the carbohydrate reserves of the muscle, the relaxation induced via adrenergic β receptors was inhibited more rapidly than that induced via α receptors. Theophylline and puromycin, which inhibit the enzymatic hydrolysis of cyclic AMP, potentiated selectively the β receptors mediated relaxation.

The mechanism or mechanisms by which catecholamines induce a relaxant effect in smooth muscle are under discussion.

Many different possible explanations for this effect have been presented (review, Lundholm, Mohme-Lundholm and Svedmyr 1966).

Mohme-Lundholm (1953) suggested that stimulation of the carbohydrate metabolism with a resulting increase in lactate production was of importance for the relaxing effect. Burnstock (1958) and Bülbring (1960) considered that stimulation of the Na—K pump in the cell membrane as a result of stimulation of the energy production was the cause of the hyperpolarization, inhibition of the electrical activity of the cell membrane and the relaxation induced by adrenaline in taenia coli

from the guinea pig Burnstock *et al* (1963) found that the magnitude of the relaxant effect of adrenaline in taenia coli from the guinea pig was dependent upon the extracellular K-concentration. They suggested that adrenaline selectively increased the permeability of the cell membrane to K and thereby induced hyperpolarization

Ahlquist and Levy (1959) demonstrated in experiments on dogs that the relaxant effect of catecholamines on the intestine *in situ* was produced via stimulation of both adrenergic α and β receptor mechanism These results were later verified by Furchgott (1960) and Buchnell and Whitney (1964) on isolated intestine from the rabbit and man respectively

In the depolarized guinea pig taenia coli cooled to a temperature that abolished the rythmic activity noradrenaline increased the permeability to K without influencing the Na permeability This effect was inhibited by adrenergic α receptor blockade It was not produced by isoprenaline or inhibited by an adrenergic β receptor blocking agent (Jenkinson and Morton 1967) They suggested that the α receptor mediated relaxation was due to a selective increase in the permeability to K

In experiments on taenia coli from guinea pigs under more physiological conditions—polarized muscle at 37° C—Bulbring *et al* (1966) and Setekleiv (1967) could find no regular relationship however between a relaxant effect of adrenaline and an increased efflux of K They showed that adrenaline increased the extracellular fluid space and increased the efflux of Na They assumed that an increased permeability to K could not alone explain the relaxant effect of adrenaline and suggested that fixation of Ca to the cell membrane and/or stimulation of an electrogenic Na pump induced this effect

Schild (1966) has shown that the catecholamines also relax completely K depolarized smooth muscle in which an effect via changes of the membrane potential seems improbable Since this relaxant effect could be influenced by the extracellular Ca^{++} concentration Schild (1967 suggested that the catecholamines can induce a relaxant effect by reducing the intracellular Ca concentration

We consider it probable that the partly contradictory nature of these explanations for the relaxant effect of catecholamines in smooth muscle may be due to the fact that the mechanism for the relaxant effect is different if it is induced via stimulation of adrenergic α receptors from that when β receptors are the mediators

Our first task was to find a smooth muscle preparation which was equipped with well developed relaxant mechanisms induced by adrenergic receptors of both the α and the β type Taenia coli from the rabbit was found to be suitable while in taenia coli from the guinea pig the mechanism mediated by adrenergic α receptors predominated We considered it probable *a priori* that the relaxation mediated via adrenergic α receptors was dependent upon a change in ionic permeability of the cell membrane We therefore attempted in different ways to influence the ionic gradients across the cell membrane and to study the way in which these measures affected the relaxant effect It seemed probably that the relaxation induced via

adrenergic β-receptors was the result of a stimulation of adenyl cyclase and a metabolic process induced via cyclic AMP We therefore studied the way in which drugs which have been reported to affect the cyclic AMP metabolism influenced the relaxant effect induced via adrenergic β-receptors

Method

The experiments were performed on taenia coli from rabbits and guinea pigs The animals were killed by a blow on the neck and the taenia coli was dissected free from the intestine The muscle preparations which were about 20 mm long, were mounted on special holders as described by Lundholm and Mohme Lundholm (1966) In this holder the distance between the points of attachment of the preparation could be varied and the muscular tension could be recorded by a tension recording transducer (FT 03) on a Grass polygraph The preparation and holder were then immersed in a bath containing 30 ml Krebs Henseleits bicarbonate buffer at 37° C The composition of the suspension solution in mM/l was 120 NaCl, 4 7 KCl 2 5 $CaCl_2$ 1 2 $MgSO_4$ 25 $NaHCO_3$ 5 6 glucose The suspension solution was gassed with 95 % O_2+5 % CO_2 the pH then being 7 4 In the experiments in which the preparations were depolarized with K ions the composition of the suspension solution in mM/l was 124 7 KCl, 2 5 $CaCl_2$ 1 2 $MgSO_4$ 1 2 KH_2PO_4 25 $KHCO_3$, 5 6 glucose

When the preparation had been allowed to become stabilized during 30—60 min in the organ bath l phenylephrine was added in a concentration of 2.5×10^{-6} g/ml, whereupon the muscle relaxed completely The preparation was then stretched gradually until a basal tension in the relaxed state of 0 2 g was induced after which the phenylephrine was washed away The length thus attained by the muscle should correspond to the fully relaxed length (L_0) (Aaberg and Axelsson 1966)

When the length of the preparation had been adjusted its tension was increased so the relaxant action of the catecholamines became more marked In most test the muscle was contracted by carbaminoylcholine (1×10^{-7} g/ml) 10 15 min before the addition of catecholamines In this concentration carbaminoylcholine produced a constant increased tension level from which it was easy to judge the extent and duration of the relaxant action of the catecholamines (Fig 7B We also increased the tension by adding $BaCl_2$ in a final concentration of (5×10^{-4} M) or KCl (1.3×10^{-2} M) to the bath In these concentrations both ions predominantly increased the rhythmic contractions of the preparation but produced only a slight constant increase of the tension The relaxant action of the catecholamines was however under different experimental conditions independent in which manner the tension had been increased

Material The catecholamine derivatives used all of which were l isomers were as follows l phenylephrine chloride (Neosynephrine® Winthrop) l isoprenaline bitartrate (Cilag Chemi), l adrenaline bitartrate) (ACO) l noradrenaline bitartrate (Nor Exadrin® Astra) The following adrenergic blocking agents were used Dl sotalol (MJ 1999 Mead Johnson and dibenamine Carbaminoylcholine chloride Karbacholin® ACO) was used Other drugs used were K strophanthoside (Strophosid® Sandoz) desoxycorticosterone acetate (DOCA® Pharmacia) theophyllinamine (Theophyllamin® ACO and puromycin (Sigma grade II All doses have been given in g/ml on free base In Fig 3 however the molar concentration of the catecholamine derivative has been given

Results

Relaxation induced via adrenergic α and β-receptors in taenia coli from rabbit

According to Ahlquist and Levy (1959) phenylephrine induces a selective adrenergic α receptor stimulation and isoprenaline a selective β receptor stimulation in intestinal muscle from the dog Taenia coli from the rabbit contracted by carbamylcholine (1×10^{-7} g ml began to be relaxed by phenylephrine in a concentration of 2×10^{-8} g ml With an increasing concentration the effect became more protracted and stronger Fig 1 Different adrenergic α receptor blocking agents were tested for their capacity to inhibit the effects of phenylephrine Both phenoxybenzamine and dihydroergotamine had in themselves a relaxing effect in a concentration which only partially blocked the effect of phenylephrine This com

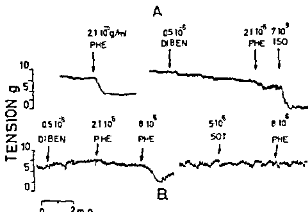


Fig 1 Taenia coli from the rabbit suspended in Krebs Henseleit bicarbonate buffer at 37° C The muscle was contracted with carbamylcholine (1×10^{-7} g/ml) 10—15 min before the addition of l phenylephrine (= PHE) or l isoprenaline (= ISO)

A Relaxant effect of phenylephrine ($2\ 1\times10^{-6}$ g/ml) Pretreatment with dibenamine (= DIBEN) ($0\ 5\times10^{-6}$ g/ml) blocked the relaxant effect of phenylephrine almost completely Isoprenaline (7×10^{-9} g/ml) still had a relaxant effect

B Dibenamine almost completely blocked the effect of phenylephrine in a low concentration but not in a high concentration A combination of sotalol (SOT) and dibenamine completely blocked the effect of the higher dose of phenylephrine

plicated the evaluation of the extent to which a decreased relaxant effect of phenylephrine was due to a true blockage of the effect or to the fact that the tension level of the muscle was reduced Dibenamine in a concentration of $0\ 5\times10^{-6}$ g/ml completely blocked the effect of phenylephrine, however, ($\leqslant 2\ 1\times10^{-6}$ g/ml) without itself influencing the muscular tension (Fig 1) In a higher concentration (8×10^{-6} g/ml), however, phenylephrine was able to break through the dibenamine blockade A further increase of the dibenamine concentration did not counteract the relaxant effect of phenylephrine in a high concentration The effect could be blocked however, by a combination of dibenamine ($0\ 5\times10^{-6}$ g/ml) and the adrenergic β receptor blocking agent sotalol (5×10^{-6} g/ml)

The threshold concentration for the relaxant effect of l-isoprenaline in taenia coli from the rabbit was 2×10^{-9} g/ml The relaxant effect of isoprenaline generally had a somewhat longer latency time than that of phenylephrine The effect of isoprenaline in a dose of $\leqslant 7\times10^{-9}$ g/ml was inhibited completely by sotalol in a con

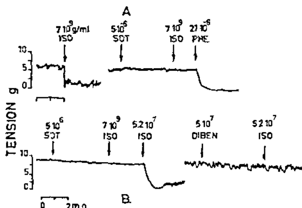


Fig 2 Taenia coli from the rabbit.

A The relaxant effect of l isoprenaline was completely blocked by sotalol Sotalol did not block the effect of phenylephrine

B Sotalol (5×10^{-6} g/ml) blocked the effect of *isoprenaline* in a low (7×10^{-9} g/ml) but not in a high ($5\ 2\times10^{-7}$ g/ml) concentration A combination of sotalol and dibenamine completely blocked the effect of *isoprenaline* in a high concentration

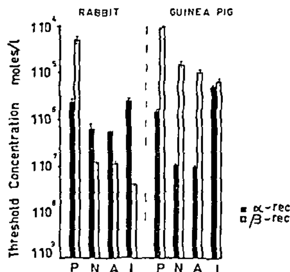


Fig. 3. Mean ± S.E.M. of the threshold concentration of different catechol amine derivatives which relaxed taenia coli from the rabbit or guinea pig by selective stimulation of adrenergic α or β receptors. Concentration on a logarithmic scale in moles/l. P = l-phenylephrine, N = l-noradrenaline, A = l-adrenaline, I = l-isoprenaline, n = 6–12.

centration of 5×10^{-6} g/ml, but not by dibenamine (Fig. 2). Its effect when used in a higher concentration ($\geqslant 5.2 \times 10^{-7}$ g/ml) could not be completely blocked by sotalol but by a combination of sotalol and dibenamine (Fig. 2).

The results of these experiments indicate that phenylephrine in a concentration of $\leqslant 2.1 \times 10^{-6}$ g/ml induced a relaxant effect via stimulation of adrenergic α receptors. In a higher concentration the relaxant effect was induced via a combination of α and β receptor stimulation. Isoprenaline in a low concentration ($< 3.7 \times 10^{-8}$ g/ml) stimulated adrenergic β receptors, while in a higher concentration it also stimulated α receptors. In view of these results it seemed of interest to attempt to determine to what extent other catecholamines such as adrenaline and noradrenaline induced a relaxant effect in taenia coli from the rabbit via stimulation of adrenergic α and β receptors. The threshold concentration for the relaxant effect of adrenaline was about 2×10^{-8} g/ml. This effect was blocked completely by sotalol but not at all by dibenamine. The effect of adrenaline in a concentration of 1×10^{-7} g/ml was inhibited only by a combination of sotalol and dibenamine.

The threshold concentrations of noradrenaline which stimulated α and β receptors were almost identical with those for adrenaline. In Fig. 3 the mean (n = 6–12) molar threshold concentrations of the studied catecholamines with specific α or β receptor stimulating effects in taenia coli from the rabbit are given. These results show that both adrenaline and noradrenaline in their lowest active concentration induced a relaxant effect via stimulation of adrenergic β receptors.

The experiments described above were performed during the months September–October. In further experiments it was found that there was a seasonal variation in the threshold concentration for the relaxant effect mediated via adrenergic β receptors. Thus the threshold concentration for the relaxant effect of isoprenaline was about 4 times lower during the summer than during the winter months. The muscle

content of adenosine triphosphate also exhibited seasonal variations with a minimum during the summer and a maximum during the winter (Andersson and Mohme-Lundholm 1969). These variations will be the subject of further studies.

Relaxation induced via adrenergic α- and β-receptors in taenia coli from guinea pigs

We considered it of interest to study also the relative importance of adrenergic α- and β-receptors for the relaxant effect of the catecholamines in taenia coli from the guinea pig since several studies on the mechanism for this effect have been carried out on this preparation. Using an identical method to that described above for taenia coli from the rabbit, the threshold concentrations of the different catecholamines for relaxation mediated by α- and β-receptors in taenia coli from the guinea pig were determined.

As can be seen in Fig. 3, the threshold concentration for the relaxant effect of phenylephrine induced via α-receptors was almost the same in taenia coli from the guinea pig as in that from the rabbit. Isoprenaline was only active in high concentrations in taenia coli from the guinea pig, since the threshold concentration for β-receptor stimulation was high in this muscle, a finding which also held for the other catecholamine derivatives. On the other hand, the threshold concentration for the relaxant effect induced via α-receptors was lower. In taneia coli from the guinea pig the relaxation induced via adrenergic α-receptors was predominantly stimulated by catecholamines, while the β-receptor mechanism was considerably less sensitive. In this respect there seems to be a difference between taenia coli from the rabbit and from the guinea pig.

The effect of K^+ ions and treatment by cold, digitalis glycosides and desoxycorticosterone on relaxation induced via α- and β-receptors in taenia coli from the rabbit

If the relaxant effect induced via α-receptor stimulation was dependent upon a polarized cell membrane and on the occurrence of ionic gradients across this membrane, measures which decreased the concentration gradient or influenced the ionic permeability of the cell membrane should inhibit the effect of α-receptor stimulation.

K^+ ions. An increase in the extracellular concentration of K^+ tends to decrease the ionic gradients of both K^+ and Na^+ across the cell membrane and to depolarize this membrane (Evans, Schild and Thesleff 1958). When the Na^+ ions in Krebs-Henseleit bicarbonate buffer were replaced by K^+ ions (145 meq/l), the relaxant effect of phenylephrine was blocked, while the effect of isoprenaline persisted, though weakened. The effect of isoprenaline was blocked completely by sotalol (Fig. 4).

K-strophanthin. Digitalis glycosides block the Na^+–K^+ pump in the cell membrane and decreases the K^+-content but increases the Na^+ content of smooth muscle (Daniel 1964). When taenia coli was treated with K-strophanthin in a concentration of 1×10^{-4} M, the relaxant effects of both phenylephrine and isoprenaline occurred initially, but after about 100 min the effect of phenylephrine was completely blocked while

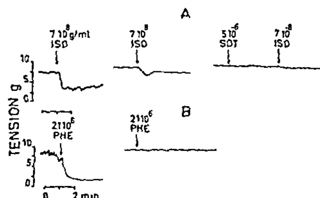


Fig. 4. Taenia coli from the rabbit. The influence of an increased extracellular K^+ concentration (145 meq/l) on the relaxant effects of isoprenaline and phenylephrine.
A. Relaxant effect of isoprenaline on taenia coli contracted with carbamylcholine (1.5×10^{-7} g/ml), the suspension solution was then changed to one containing 145 meq/l K^+ (without carbaminoylcholine) and relaxation with isoprenaline was again induced. The relaxant effect of isoprenaline in a K^+ rich solution was completely blocked by sotalol.
B. In taenia coli contracted with carbaminoylcholine the relaxant effect of phenylephrine was apparent but not when the preparation had been contracted with K^+ ions in a high concentration (145 meq/l).

that of isoprenaline persisted (Fig. 5 A). The long latency period before the occurrence of the blockade of the *a* receptor-stimulated relaxation indicated that it was probably not the blockade of the Na—K pump as such which inhibited the relaxant effect, but that this blockade did not occur until the ionic gradients across the cell membrane had decreased.

Treatment with cold. When taenia coli was stored for 24 hrs at +2° C in oxygenated Krebs-Henseleit bicarbonate solution and then suspended in the same solution for 20 min at 37° C, isoprenaline still induced a relaxant effect which could be blocked with sotalol.

Phenylephrine, on the other hand, had no effect. After suspension for 120 min at 37° C, however, the relaxant effect of phenylephrine returned (Fig. 5 C). This is in agreement with results of Lum, Hermann and Gaunt (1967).

Desoxycorticosterone. According to Bass, Hurwitz and Smith (1964), desoxycorticosterone inhibited the increase in the efflux of K^+ induced by pilocarpine in smooth muscle. It seemed of interest, therefore, to determine whether desoxycorticosterone influenced the relaxant effect of catecholamines. It is evident from Fig. 5 B that the relaxant effect of phenylephrine on taenia coli was completely blocked when the preparation had been treated for 60 min with desoxycorticosterone in a concentration of 1×10^{-5} moles/l. On the other hand, the effect of isoprenaline was not inhibited. When the desoxycorticosterone had been washed away, the relaxant effect of phenylephrine returned.

The influence of substrate depletion on the relaxant effect of phenylephrine and isoprenaline

According to Bueding and Bülbring (1964), the catecholamines also relax a "glycogen free" taenia coli from the guinea pig. These authors consider that this finding contradicts the possibility that stimulation of the carbohydrate metabolism is of importance for the relaxant effect of the catecholamines. In a series of experiments

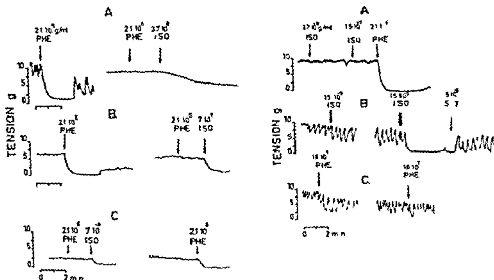


Fig 5 Taenia coli from the rabbit The effect of K-strophanthin desoxycorticosterone and treatment with cold

A The relaxant effect of phenylephrine was first tested after which the preparation was treated with K strophanthine (1×10^{-4} M) during 100 min The effect of phenylephrine was blocked while that of isoprenaline persisted

B When the relaxant effect of phenylephrine had been tested desoxycorticostrone acetate was added in a concentration of 1×10 M After 60 min treatment the effect of phenylephrine was blocked, while that of isoprenaline persisted

C The preparation was suspended during 24 hrs in oxygenated Krebs Henseleit bicarbonate buffer at +4° C 20 min after warming of the buffer solution to 37 C and the addition of carbaminoylcholine, phenylephrine induced no relaxant effect isoprenaline induced such an effect, however After 120 min at 37° C the relaxant effect of phenylephrine returned

Fig 6 Taenia coli from the rabbit The effect of substrate depletion' and theophylline on the relaxant effects of isoprenaline and phenylephrine

A The preparation was suspended during 90 min in glucose free Krebs Henseleit bicarbonate solution gassed with N_2 The suspension solution was then gassed with 95 % O_2 + 5 % CO_2 and 11.5×10^{-3} M Na-pyruvate was added The preparation was contracted with carbaminoylcholine Isoprenaline in the dose region of 3.7×10^{-8} – 1.5×10^{-7} g/ml had no relaxant effect but such an effect was still induced by phenylephrine

B Isoprenaline in a concentration of 1.5×10^{-8} g/ml had no definite relaxant effect 10 min after the addition of theophylline (5×10^{-5} g/ml) isoprenaline in the same concentration induced a markedly potentiated relaxant effect After addition of sotalol the rhythmic activity returned.

C The relaxant effect of phenylephrine was not potentiated by theophylline (5×10^{-5} g/ml) added 10 min before

In the present study, a "glycogen free muscle preparation was produced as described by Bueding and Bulbring (1964) Taenia coli from the rabbit was stored for varying lengths of time under anaerobic conditions in glucose free solution 0.1 % Na pyruvate was then added as substrate and the solution was bubbled with 5 % CO_2+95 % O_2 When tension had been induced by carbaminoylcholine, the relaxant effects of phenylephrine and isoprenaline were tested As is shown in Fig 6 A after 2 hrs under anaerobic conditions the relaxant effect of isoprenaline had com-

Fig. 5. Taenia coli from the guinea pig. The influence of an unresponsive ... on the relaxant effects of isoprenaline and phenylephrine.

A. ... effect of isoprenaline on taenia coli contraction ... suspension solution was ... changed to one containing 1... meq/l K ... and relaxation with isoprenaline was ... The relaxant effect of isoprenaline ... was completely blocked ...

B. In taenia coli contracted ... effect of phenylephrine was apparent but not when the ... K ... a high concentration (1... meq/l

... Fig. 5 A. The long latent period before the occur... relaxation indicated that it was ... the Na—K pump as such which inhibited the re... blockade did not occur until the ionic gradients across ... decreased.

... When taenia coli was stored for 24 hrs at +2 C in ... bicarbonate solution and then suspended in the same ... at 37° C isoprenaline still induced a relaxant effect which could ...

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The influence of ... substrate depletion on the relaxant effect of phenylephrine and isoprenaline

According to Bueding and Bülbring (1964) the catecholamines also relax a glycogen-free taenia coli from the guinea pig. These authors consider that this finding contradicts the possibility that stimulation of the carbohydrate metabolism is of importance for the relaxant effect of the catecholamines. In a series of experiments

Bulbring 1967) Imidazole in a concentration of 2.4×10^{-4} g/ml had a direct contractile effect on taenia coli from the rabbit and also reduced the relaxant effect of both isoprenaline and phenylephrine (Fig 7 B C)

Discussion

In taenia coli from the rabbit the catecholamines had a relaxant effect which was induced via two different mechanisms These differed from each other in several respects One of them was stimulated by a low concentration of phenylephrine which is a catecholamine derivative that possess predominantly adrenergic α receptor stimulating properties according to the classification of Ahlquist (1948) The effect of phenylephrine in a low concentration was blocked by dibenamine a drug with predominantly α receptor blocking properties Another mechanism with a relaxant effect was stimulated by a low concentration of isoprenaline which is a catecholamine derivative with predominantly adrenergic β receptor stimulating properties, the effect of isoprenaline was blocked selectively by sotalol which is an adrenergic β-receptor blocking agent with no or only negligible effects of its own (Aaberg *et al* 1969) In higher concentrations both phenylephrine and isoprenaline had a relaxant effect via combined stimulation of α and β receptors These effects could be blocked completely by a combination of adrenergic α- and β receptor blockade It is clear therefore that phenylephrine and isoprenaline acted as specific stimulators of α and β receptors only in low concentrations

In taenia coli from the rabbit adrenaline and noradrenaline in their lowest active concentration (2×10^{-8} g/ml) stimulated adrenergic β receptors In a concentration about 5 times higher adrenergic α receptors were also stimulated In taenia coli from the guinea pig the threshold concentration 2×10^{-8} g ml for the relaxant effects of adrenaline and noradrenaline was almost identical with that in taenia coli from the rabbit In taenia coli from the guinea pig however the relaxation was induced via adrenergic α receptors, while the threshold concentration for a β receptor stimulating effect was about 100 times higher It would seem as if the relaxation mechanisms in the two animal species had adapted their sensitivity to the same physiological concentration of adrenergic transmittor substance but that in the evolution of the two species different mechanisms had been developed for mediating the relaxant effect of the hormone

The relaxation mechanisms stimulated via adrenergic α and β receptors differed not only in their capacity for being stimulated or blocked by various drugs but also in the way they were influenced by the experimental conditions

Measures which tended to eliminate the ionic gradients across the cell membrane and depolarize this membrane such as an increase in the extracellular K concentration digitalis glycosides treatment of the muscle with cold and also with desoxycorticosterone which has been reported to inhibit the permeability of the cell membrane to K blocked the relaxant effect induced via adrenergic α receptors while that induced via adrenergic β receptors persisted On the other hand drugs which can be assumed to have blocked the enzymatic hydrolysis of cyclic AMP,

such as theophylline and puromycin, potentiated the relaxant effect induced via β receptors without influencing that induced via α receptors. Sotalol, which specifically blocked the relaxation induced via β receptors, has been shown in skeletal muscle to inhibit the stimulation by adrenaline of cyclic AMP production (Bowz and Lundholm 1969). On reduction of the carbohydrate reserves of the muscle the relaxation induced via β receptors was also inhibited at an earlier stage than the effect induced via α receptors. Both mechanisms of relaxation were blocked by imidazole, which has been reported to stimulate the enzymatic hydrolysis of cyclic AMP (Butcher and Sutherland 1962). In view of this finding it can be asked whether the effect of imidazole can be attributed only to a specific action on the cyclic AMP metabolism and cannot, for example, be due to the fact that imidazole had itself increased the muscular tension.

The differences in the properties of the two relaxation mechanisms are not limited only to those mentioned above. In further experiments it was found that the latency period for a relaxant effect induced via α receptors was shorter than that for β receptor induced relaxation, and that the relaxation was more complete after α induced than after β induced stimulation. Differences were also observed in the metabolic processes which accompanied the two types of relaxation (Andersson and Mohme-Lundholm 1968).

All these findings indicate that the catecholamines can induce a relaxant effect in smooth muscle via two different mechanisms. It would seem to be of great importance to take this into consideration in further studies of the mechanisms by which catecholamines relax smooth muscle. In previous investigation it would seem to have been either an α- or β receptor or a combined α and β receptor stimulation that induced the relaxation. It is difficult to determine in retrospect which of the two mechanisms is of the greatest importance in different cases.

In our studies factors which tended to reduce the ionic gradients across the cell membrane blocked the relaxant effect induced by stimulation of adrenergic α receptors. Our experiments support the assumption that a stimulation of α receptors leads to a change in the permeability of the cell membrane to a certain ion or ions. The question of whether the relaxant effect of α receptor stimulation can be attributed only to an increase in permeability to K^+ ions, as has been suggested by Jenkinson and Morton (1967), cannot be answered from our studies. Treatment both with cold and with digitalis glycosides induces a decrease in both the Na^+ and the K^+ gradient across the cell membrane of smooth muscle (Daniel 1964), which is also produced by an increase in the extracellular K^+ concentration. Desoxycorticosterone can decrease the Na^+ content of skeletal muscle (Withrow and Woodbury 1959) and therefore, without further investigations, it cannot be assumed that this corticosteroid influenced only the permeability to K^+.

The relaxant effect induced via adrenergic β receptors was blocked on reduction of the carbohydrate reserves of the muscle; it was also potentiated by drugs (theophylline and puromycin) which decrease the enzymatic hydrolysis of cyclic AMP. These results indicate a relationship between a stimulation of metabolic

processes and the relaxation process induced via adrenergic β receptors This relationship has been studied more closely in another investigation (Andersson and Mohme Lundholm 1969)

We are indepted to Miss Gun Abrahamson for technical assistance Financial support has been given by the Swedish State Medical Research Council (B68 14X 2080 03) and the Swedish National Association against Heart and Chest Diseases

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exterior through a stab wound in the abdominal wall by a plastic coated stainless steel cannula (Condon and Harkins 1962) Vagally denervated *antral pouches (AP)* were prepared in two dogs in a manner described earlier (Rheault et al 1965) The antral pouch was brought to the exterior by means of a mucocutaneous fistula or by an indwelling plastic coated stainless steel cannula In two dogs, *jejunal pouches (JP)* were prepared as a Thiry fistula of approximately 20 cm intestine from the upper part of the jejunum (Markow... nie 1964) These pouches were

The animals were kept in sep... cornflour, with the addition of ...

... which the animals were under antibiotic treatment

Preparation of extracts

Secretion from the antral pouches was collected by rinsing with 0 9 % NaCl solution through a rubber balloon catheter after the injection of pilocarpine, 1 ml subcutaneously of 0 001 mg/ml The secretion was collected by inserting a rubber balloon catheter, and by washing with 0 9 % saline Heidenhain pouch secretions were collected after subcutaneous injection of 1 mg of histamine acid diphosphate The juice from jejunal pouches was collected in a manner similar to the antral washings No stimulation was attempted in the case of the jejunal pouches The secretions were drained into sterilized bottles The juices were all filtered through glass wool and dialyzed in di...

... All experiments the extracts were given in doses of 1 mg/kg b w

Assay methods

The different extracts were assayed for inhibitory activity according to the method reported by Code, Blackburn, Livermore and Ratke (1949b) A continuous stimulation of gastric HP secretion was applied at approximately 50 % of maximal secretion by giving histamine acid phosphate slowly i v through an infusion pump Solutions of histamine acid phosphate were made up in 0 9 % NaCl immediately before administration The dogs received doses of 200—300 μg/hr, (73—109 μg histamine base) delivered i v by a constant infusion pump at a rate of 15 ml/hr The dose of histamine to be used in each animal was determined according to dose-response curves in preliminary experiments, and these doses were used in all studies reported here

At each test the animals were taken to the Pavlov stand after overnight fasting for 12—18 hrs with only water *ad libitum* and underwent approximately an hour's observation to ensure

... tion varied no more than 0 5 ml in 3 consecutive 15-min periods, the extracts were injected through another vein by a single i v injection lasting about 30 sec The antral extracts were assayed a total of 22 times in 5 dogs and the fundic extracts 21 times in 4 dogs, while the jejunal extracts were tested 15 times in 3 dogs

Collection of gastric juice

The gastric juice was collected from the HP by gravity drainage into graded centrifuge glasses fitted to the cannula from the pouch by means of a plastic adapter The samples were collected at 15 min intervals, care being taken to empty the pouch completely by inserting a thin rod in the cannula to clear the passage of mucus plugging

Volumes of gastric juice

The volumes of each sample were measured directly in the graded centrifuge tube, and read to the nearest 0 1 ml

Titration for acid

Each sample of gastric juice was titrated against 0 01N NaOH to pH 7 0 with neutral red as indicator

Rectal temperatures
In all assays, thermometer (original USCO Fieber thermometer) recorded rectal temperatures of each animal to the nearest 0 1° C every 15 min

Histamine in gastric juices and extracts
Two samples of AP juice (5 ml) and HP juice (10 ml) taken before and after dialysis for 48 hrs as described above, and samples of 2 mg each from dialyzed and freeze-dried extracts of AP, HP and JP juice were analyzed for histamine contents according to the method of Shore, Burkhalter and Cohen (1959), utilizing a Farrand Spectrophotometer combined with a Varian recorder

Analysis of the results
The secretory values are shown in all figures as per cent secretory output during the control period The 100 % level is the average acid output of three 15-min periods immediately preceding the injection of test extracts. In a previous study (Semb 1966*b*), the response to i v infusion of histamine by a constant delivery pump (Harvard pump) was studied in 6 dogs on 14 occasions The coefficients of variation have been calculated as being between 10 1 and 20 8 % The experimental model used in the present study is the same as that mentioned earlier, and preliminary tests with some of the animals used in this study show that the present variability is of the same order of magnitude These variations are also in keeping with the findings of other authors (Code *et al* 1949*a*, Shapira *et al* 1960) For this reason it seems justifiable to consider significant changes in secretion as being 21 % or more in comparison to control levels. For each type of experiment, the maximal change in gastric acid secretion as per cent of control level has been plotted against maximal change in rectal temperature after the injection of test substances

The rectal temperatures in each 15-min period are expressed as changes from the rectal temperature measured in the 15-min period immediately preceding the injection of test substances

Results

Injection of antral extracts

Antral juice extracts were injected i v in a total of 22 expts on 5 dogs In all dogs, significant inhibition of the *gastric acid secretion* was observed altogether in 17 of 22 expts After the injection of extracts, inhibition occurred within 75 min in all tests, the average maximal inhibition being 63 6 % (range 26—90 % of control) In

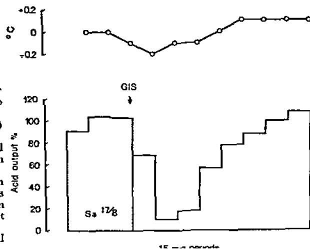


Fig 1 Gastric acid output as per cent of control in response to continuous i v infusion of histamine in dog Sa before (shaded) and after (open) the i v injection of 1 mg/kg b w of antral juice extract (GIS, arrow) Each bar represents one 15 min period Rectal temperature changes in comparison to pre injectory levels are indicated by open circles In this experiment a significant reduction in acid output and in significant changes in rectal temperatures are shown

nie 1964). These pouches were also taken to the exterior as mucocutaneous fistulae

The animals were kept in separate cages and were all maintained on a diet of meat, fish and cornflour, with the addition of vitamins and protein powder NaCl and KCl were added to the food to counteract the electrolyte loss from the cannulated pouches The weights of all animals were well maintained Before experiments were started, the animals were allowed at least one month to recover from surgery From time to time suppuration developed around the cannula, and this was treated with Penicillin 600,000 U and Streptomycin 1 g daily for 5 days Tests were not run in those periods when the animals were under antibiotic treatment

Preparation of extracts

Secretion from the antral pouches was collected by rinsing with 0 9 % NaCl solution through a rubber balloon catheter after the injection of pilocarpine, 1 ml subcutaneously of 0 001 mg/ml The secretion was collected by inserting a rubber balloon catheter, and by washing with 0 9 % saline Heidenhain pouch secretions were collected after subcutaneous injection of 1 mg of histamine acid diphosphate The juice from jejunal pouches was collected in a manner ... ches ... lass ... C), ... dic ... in ... of ... en

Assay methods

The different extracts were assayed for inhibitory ... reported by Code, Blackburn, Livermore and Ratke (1949b ... P ... secretion was applied at approximately 50 % of ... d ... phosphate slowly i v through an infusion pump ... e made up in 0 9 % NaCl immediately before administration The dogs received doses of 200—300 μg/hr, (73—109 μg histamine base) delivered i v by a constant infusion pump at a rate of 15 ml/hr The dose of histamine to be used in each animal was determined according to dose-response curves in preliminary experiments, and these doses were used in all studies reported here

At each test the animals were taken to the Pavlov stand after overnight fasting for 12—18 hrs with only water *ad libitum*, and underwent approximately an hour's observation to ensure that there was no secretion of acid from the HP Following the observation period infusion of histamine was then started through a vein cannulated by means of a plastic needle (Braunule®, Braun Melsungen, Germany) connected to the infusion pump The infusion was maintained for at least six 15 min periods after the HP secretion reached a secretory plateau When secretion varied no more than 0 5 ml in 3 consecutive 15-min periods, the extracts were injected through another vein by a single i v injection lasting about 30 sec The antral extracts were assayed a total of 22 times in 5 dogs and the fundic extracts 21 times in 4 dogs, while the jejunal extracts were tested 15 times in 3 dogs

Collection of gastric juice

The gastric juice was collected from the HP by gravity drainage into graded centrifuge glasses fitted to the cannula from the pouch by means of a plastic adapter The samples were collected at 15 min intervals, care being taken to empty the pouch completely by inserting a thin rod in the cannula to clear the passage of mucus plugging

Volumes of gastric juice

The volumes of each sample were measured directly in the graded centrifuge tube, and read to the nearest 0 1 ml

Titration for acid

Each sample of gastric juice was titrated against 0 01N NaOH to pH 7 0 with neutral red as indicator

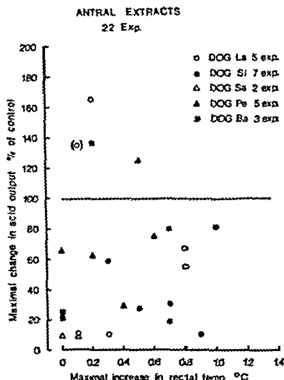


Fig 3 The maximal change in gastric acid output as per cent of control and the maximal increase in rectal temperature after the injection of antral juice extracts 22 expts in 5 dogs The individual results are indicated by different symbols for each dog The shaded area indicates the limits of significance

(range 28—77 % of control) The acid secretion returned to pre injectory levels within three 15 min periods in most experiments where inhibition was demonstrated In 10 expts, a significant stimulation of gastric secretion was seen 15—75 min after the injection (Fig 5) The average maximal stimulation was 53 6 % (range 27—152 % above control) The stimulation lasted for 3 or more 15 min periods in all but 1 expt In 3 expts on 2 dogs there was an initial depression of gastric acid secretion after the injection followed by significant stimulation

In 13 of the 21 expts with fundic extracts the *rectal* temperatures rose gradually but in only 4 was the rise 0 5° C or more The maximal changes in rectal temperature occurred at times varying from 30 min to 135 min after the injection of fundic extracts In the remainder of the experiments there was either no elevation of rectal temperatures or a reduction of 0 1—0 5° C No clear time relationship was established between the acid gastric secretion and rectal temperature in two of the tests there was significant inhibition and in two others significant stimulation of gastric secretion before significant rise in temperature was observed However, comparing maximal changes in rectal temperature and in gastric acid secretory change irrespective of time relationship in all tests increasing gastric acid secretion was demonstrated with increasing rectal temperature after the injection of fundic extracts (Fig 6)

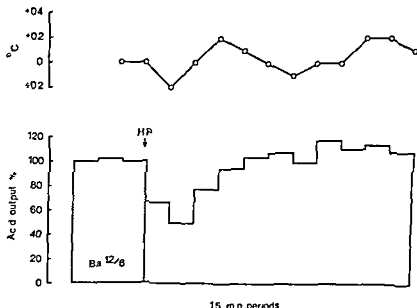


Fig 4 Gastric acid output as per cent of control in response to continuous i v infusion of histamine in dog Ba before (shaded) and after (open) the i v injection of 1 mg/kg b w of fundic juice extracts (HP, arrow) Conventions as in Fig 1 In this experiment significant reduction in gastric acid output with insignificant elevation of rectal temperature are seen

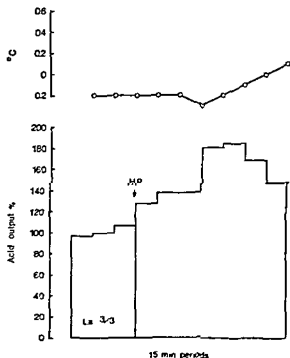


Fig 5 Gastric acid output as per cent of control in response to continuous i.v infusion of histamine in dog La. Conventions as in Figs 1 and 4 In this experiment a significant elevation of gastric acid output and an insignificant elevation of rectal temperature are seen

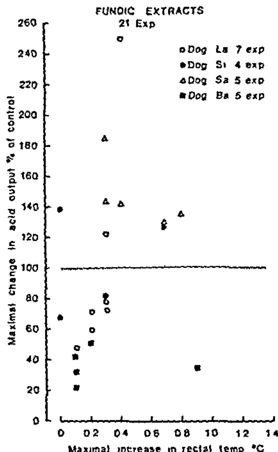


Fig 6 The maximal change in gastric acid output as per cent of control and the maximal increase in rectal temperature after the injection of fundic juice extracts 21 expts in 4 dogs The individual results are indicated by different symbols for each dog The shaded area indicates the limits of significance

Injection of jejunal extracts

Jejunal extracts were injected i v in a total of 15 expts on 3 dogs In all dogs, significant inhibition of the *gastric acid secretion* was observed altogether in 9 of the 15 expts (Fig 7) After the injection of extracts the inhibition occurred within 15—75 min in all tests, the average maximal inhibition being 50 6 % (range 27—93 % of control) In two of the experiments the gastric acid secretion returned to control levels within 15 min but in the remaining six the gastric acid secretion was inhibited for longer periods In three experiments, there was no significant change in gastric acid secretion and in a further 4 there was significant stimulation of the secretion (3 dogs) The stimulation was seen 15—45 min after the injection of extracts and lasted for 15—75 min The average maximal stimulation was 47 7 % (range 33—67 % above control) I 2 expts, both stimulation and inhibition were observed

The *rectal temperatures* rose gradually after the injection of jejunal extracts in 7 tests on two dogs, and all were elevated 0 5° C or more The maximal changes oc-

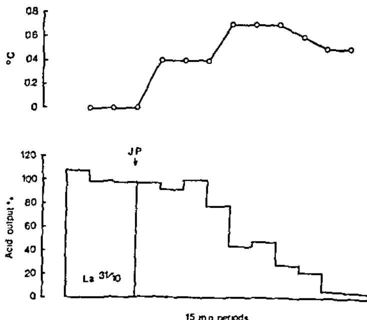


Fig 7 Gastric acid output as per cent of control in response to continuous i v infusion of histamine in dog La before (shaded) and after (open) the i v injection of 1 mg/kg of jejunal juice extracts (JP, arrow) Conventions as in Fig 1 In this experiment there is significant reduction in gastric acid output as well as a significant elevation of rectal temperatures

curred 60—90 min after the injection, and lasted for 3 or more 15 min periods Maximal gastric secretory inhibition and maximal changes in rectal temperature usually occurred in the same 15 min periods In all but one test where inhibition of acid secretion was found there was a significant elevation of rectal temperature (Fig 8) In the third dog, the rectal temperatures were not raised after the injection of jejunal juice extracts, and inhibition was found in only one of five experiments

Discussion

In testing all three types of extracts, two effects were apparent, namely inhibition and stimulation of gastric acid secretion Antral extracts showed the most pronounced inhibition in the majority of experiments (17/22), while extracts from fundic juice produced inhibition in 10 of 21 experiments, and the jejunal extracts in 9 of 15 expts

Inhibitory effects of extracts from gastrointestinal secretions have previously been observed by several investigators Inhibition has been demonstrated on i v injection of extracts of saliva (Code *et al* 1949*b*, Menguy, Masters and Gryboski 1964) gastric juice (Brunschwig *et al* 1939, Livermore and Code 1952 Hood, Code and Grindlay 1953, Semb 1966*a*, *b*), juice from jejunum and ileum (Rudick *et al* 1966*b*), and also in the thoracic duct lymph (Rudick *et al* 1966*a*) The mechanism of this inhibition is not known

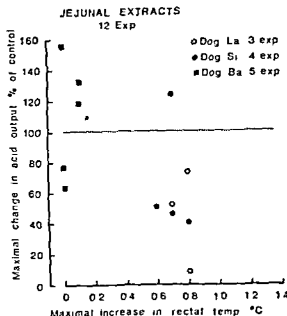


Fig 8 The maximal change in gastric acid output as per cent of control and the maximal increase in rectal temperature after the injection of jejunal juice extracts, 12 expts. in 3 dogs The individual results are indicated by different symbols for each dog The shaded area indicates the limits of significance.

Some of the earlier reports conflict with the present findings Hood, Code and Grindlay (1953) found that fundic juice extracts had little or no effect on gastric secretion in dogs, although their report contains information of secretory inhibition up to 44 % in 16 of 20 expts (range 1—44 % of control) Menguy, Masters and Gryboski (1965) found no effect on gastric secretion from the injection of fundic juice extracts prepared from dog Heidenhain pouch when tested in the pylorus ligated rat

The stimulating effects observed of some of the extracts in the present study compare favourably with earlier observations For instance Hood Code and Grindlay (1953) found stimulation in one of the 20 fundic extracts they tested Extracts of dog antral juice were shown by Baume and Nicholls (1966) to stimulate gastric secretion in the *in vitro* inverted rat stomach Stimulation of gastric acid secretion has been observed after injection of gastrone from anacid human gastric juice into the dog (Code 1966)

The reasons for the variations in results described above are unclear The extracts from the HP were autologous and those from the AP and JP were made from two dogs, and assayed in at least two dogs on different occasions Since the extracts prepared from the same dogs gave variable responses (Figs 3 6) a difference between the donors is an unlikely explanation It is well known that gastric juice contains histamine (Lorenz and Pfleger 1968) and the possibility exists that the variation in response to the extracts could be ascribed to a contamination with histamine However, samples of juice from the AP and HP were assayed for histamine content

before and after dialysis for 48 hrs, and the reduction in histamine content was found to be 94 and 86 % respectively Dialyzed and freeze dried samples of AP, HP and JP juice extracts from some of the dogs used in this study were found to contain between 0 05 and 0 12 µg histamine base per mg dry weight Lorenz and Pfleger (1968) cite studies on the sub minimal dose of histamine required to elicit gastric secretion from denervated pouches in dogs as being in the order of 0 015—0 08 µg histamine base per kg an hour injected i v Thus the possibility cannot be fully excluded that the extracts used in this study contained enough histamine to explain the stimulatory effect seen in some of the experiments However, it seems less likely that a small content of histamine, as demonstrated, should be large enough to mask the effects of an inhibitor contained in the same extract.

From the studies mentioned above and from the present study it can be concluded that extracts from the secretions of some epithelia in the gastrointestinal tract contain substances capable of both inhibition and stimulation of gastric acid secretion, the latter probably being due to small amounts of histamine

The increase in rectal temperature which is sufficient for producing inhibition of gastric secretion is not clear from earlier work Blickenstaff and Grossman (1950) never found inhibition of gastric acid secretion after the injection of pyrogens, which increased temperature less than 0 3° C in the dog Wyllie Limbosch and Nyhus (1967) studied the inhibitory effect of Pseudomonas polysaccharide pyrogen (Piromen) on histamine stimulated Heidenhain pouch secretion in 3 dogs They found *inhibition in the dogs with doses that produced increase in rectal temperatures greater* than 0 5° C Brodie and Kundrats (1964) found gastric secretory inhibition in the rat only when rectal temperatures were elevated 0 8° C or more In view of this information a change of more than 0 5° C in this study was considered as significant pertaining to its possible inhibitory effect on the stomach Inhibition and stimulation could be demonstrated with all types of extracts regardless of rectal temperature changes In the majority of experiments with inhibition with both antral and fundic extracts the rectal temperatures were below 0 5° C Inhibition of gastric acid secretion and changes in rectal temperature were not negatively correlated for any of the three types of extract in the present study

These findings suggest that inhibition of gastric secretion produced by i v injec*tion of extracts obtained from various gastrointestinal fluids cannot solely be ex*plained by the presence of pyrogens in the extracts

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Structure and Function of the Tactile Hair Receptors on the Cat's Foreleg

By

Bengt Y Nilsson

Received 18 February 1969

Abstract

Nilsson, B Y *Structure and function of the tactile hair receptors on the cat's foreleg* Acta physiol scand 1969 77 396—416

... free nerve endings and Merkel cell ... at varying ... ond to static ... ency attain a steady state whose frequency is related to the displacement amplitude by a power function ... tion velocities were determined

The major portion of the cat's skin is covered by comparatively short hairs which are associated with rapidly adapting hair follicle receptors with a high dynamic sensitivity (Adrian 1931, Brown and Iggo 1967) In addition to these hairs there are morphologically distinct tactile hairs which are recognized by their larger size and by the presence of a large blood sinus in the hair follicle A well-known type of these so called sinus hairs are the vibrissae which have been shown to be associated with slowly adapting mechanoreceptors (Fitzgerald 1940) On the cat's foreleg, just above the paw, there is a group of sinus hairs differing morphologically from the vibrissae in that the hair follicle is surrounded by a smooth pilomotor musculature and that there is a large number of Pacinian corpuscles close to the sinus These carpal sinus hairs were first studied physiologically by Nilsson and Skoglund (1965) On manual bending of the hairs very slowly adapting impulse discharges were recorded, the frequency of which was dependent on the degree of deflection The

hairs were also found to have a certain directional sensitivity, displacement in one direction increased the discharge frequency whereas movement in the opposite direction might abolish a previous spontaneous activity

The purpose of the present paper is to report a more extensive investigation of the properties of these carpal tactile sinus hairs in the cat In the first section, an account will be given of the structure of the hair follicle particular attention being paid to its innervation The main portion of the paper is concerned with the physiological properties of the hair follicle receptors of the slowly adapting type By means of a specially designed mechanical stimulator permitting displacements of the hair at varying rates and amplitudes a quantitative analysis of the dynamic and static responses thus obtained has been performed The directional sensitivity of the hair receptors has been studied in more detail and the conduction velocity in the afferent nerve fibers measured A preliminary study of hair follicle receptors of rapidly adapting type will also be presented

Methods

Electrophysiological methods Twenty-six cats weighing between 2 0 and 4 8 kg were used The animal was anesthetized with Nembutal 40 mg/kg b w i p and the anesthesia was maintained by repeated i v injections of small doses of the same drug as required The cat was kept warm by means of an infrared heating lamp and the temperature continually controlled rectally as well as subcutaneously in the vicinity of the carpal tactile hairs The rectal temperature varied between 37 and 39° C and the subcutaneous temperature between 33 and 36° C.

The forelimb of the animal was fixed with a clamp around the paw and another around the elbow joint, care being taken not to disturb the blood supply to the leg All hairs on the lower leg except the sinus hairs were closely clipped An incision was made in the skin from the elbow joint down to a point 10 mm proximal to the group of tactile hairs The superficial flexor muscles of the lower leg were removed and the ulnar nerve branch supplying the tactile hairs was prepared free and cut about 60 mm proximal to the hairs A paraffin pool was formed [illegible]

[illegible]

wire electrodes located 35—45 mm distal to the recording electrode

Mechanical stimulation For the quantitative analysis of the receptor properties a specially designed mechanical stimulator of electrodynamic type was used This stimulator represents an improved version of an electromechanical transducer of a type previously used at this department (Lindblom 1958 Shepherd and Ottoson 1965) The stimulating probe was made by gluing one end of a piece of stainless steel cannula 1 mm in diameter and 80 mm in length into the center of the cone of a Philips model AD 2300 BZ loudspeaker the voice coil impedance of which is 400 ohms Displacements of various amplitudes and velocities were obtained at the free end of the cannula by feeding the loudspeaker coil with ramp or in some cases square waveforms from a function generator designed for this specific purpose In order to reduce overshoot and ringing the loudspeaker cone was damped by mounting a metal plate in front of it the cannula being passed through a small hole in the center of the plate Due to this damping the maximal velocity was restricted to about 80 mm/sec

The unknown and varying mechanical loading presented to the stimulating probe made necessary some method of monitoring the displacement during the actual stimulating conditions Because of the small force requirements of a capacitive transducer this was chosen for recording [illegible]

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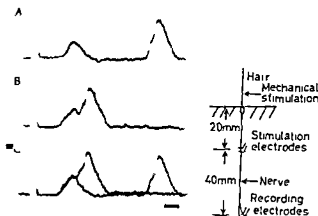


Fig 1 Determination of conduction velocity by means of the "collision method" Diagram to the right of the records illustrates arrangement of stimulating and recording electrodes as well as site of mechanical stimulation Time bar 0 2 msec Full description in text

scope for the display Within the range of displac
there was a linear relationship between the capaci
tude Stimulation amplitudes up to 450 μ were us
to 63 mm/sec Using high speed ramp waveforms
ment showed a small exponential curvature The displacement velocity was therefore established by measuring the slope of the film records between the 10 % and the 90 % points of full amplitude

The mechanical stimulator was affixed to a ball and socket joint which in turn was mounted on a very heavy and stable stand, this permitted movements of the stimulator in vertical and lateral directions

The stimulator was monitored under a dissection microscope so that the probe was just in *contact with the sinus hair under study Spontaneous activity, if present, was not or but little* affected During the stimulation the probe was moved parallel to the skin surface and at approximately right angles to the hair The contact point between probe and hair was about 2—3 mm above the skin surface

In the initial experiments a somewhat simpler mechanical stimulator of similar type was employed, consisting of a loudspeak
damping plate in front of the cone
be obtained The loudspeaker was c
S 4, and the amplitude of the dis
calibrated scale in the eyepiece T
Displacem

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(*cf* Brown and Iggo 1967) For this purpose, the hair was displaced by means of the mechanical stimulator and the sweep triggered so that the first impulse in the discharge was displayed at the right hand side of the oscilloscope screen using an expanded sweep (Fig 1 *A*) Then the nerve was excited with stimulating electrodes placed between the hair and the recording electrodes (see diagram in the figure) in such a way that the stimulus artefact and *the response were displayed* at the *left* hand side of the screen In *A*, the strength of the

the response at the right hand side of the screen (*B*)
between the stimulus artefact and the
measured and the conduction velocity
ce
airs with adjacent tissue were removed
lin were stained with Weigert s hema-

toxylin and eosin For innervation studies one preparation was treated according to Palmgren's method and others were fixed in chloral hydrate and impregnated with silver nitrate The tissue was cut serially at 4 or 8 μ

Results

Morphology of the sinus hair follicle

The main morphological features of the carpal sinus hairs were presented in a previous paper (Nilsson and Skoglund 1965) After its publication an early histological description of these hairs was found (Fritz 1909) Since in our previous paper as well as in the immediately subsequent publication (Nilsson 1969) in this series of investigations pictures showing the basic structure of the hair follicle have been reproduced only some of its characteristics will be briefly recalled below with reference to Fig 2 in which *A* shows a transverse section of the follicle through the lower portion of the blood sinus As in other types of sinus hairs the external sinus wall (*e* in the figure) is composed of strong connective tissue while the internal wall next to the hair consists of a mesenchymal tissue separated from the external root sheath by a so-called glassy membrane (*g* see also Fig 2 *D*) The lower portion of the sinus is filled with a cavernous tissue while the upper portion the ring sinus, is filled with blood From the internal mesenchymal sinus wall a 'ringwulst' projects into the ring sinus

Particular attention has been paid to the innervation of the carpal tactile sinus hairs which does not seem to have been described in more detail previously Fig 2 *A* shows a bundle of nerves (at arrow) penetrating the external sinus wall In the cavernous sinus the hair is surrounded by a large number of transversely cut nerve fibers many of which are assembled into bundles containing up to about 15 fibers which have penetrated the sinus wall at a lower level In sections at higher levels of the sinus the nerve fibers can be seen in the internal mesenchymal sinus wall just outside the glassy membrane (Fig 2 *D*) As the fibers ascend they branch the number of transversely cut fibers increases and there are fewer fibers in each bundle Fig 2 *B* shows an oblique section through the internal sinus wall at the level of the ringwulst, with a palisade of fine nerve fibers arranged around the hair These fibers are distributed symmetrically around the hair In the ringwulst no nerve fibers have been found In other sections through the internal sinus wall a complex network of winding varicose fibers can be seen (Fig 2 *C*)

In the external root sheath just inside the glassy membrane there are large cells with a clear, pale cytoplasm, so-called Merkel cells Fine nerve fibers penetrate the glassy membrane, and endings located close to these cells are visible in Fig 2 *D*

Adrenergic nerve endings have been demonstrated in the cavernous portion of the sinus and in the internal sinus wall (Fuxe and Nilsson 1965)

In the vibrissae also more complex receptor structures have been found thus *e g*, Ruffini like endings have been described (Miller *et al* 1960) as well as also lamellated corpuscles (Andres 1966) No such structures were observed in the sections of carpal tactile hairs studied here

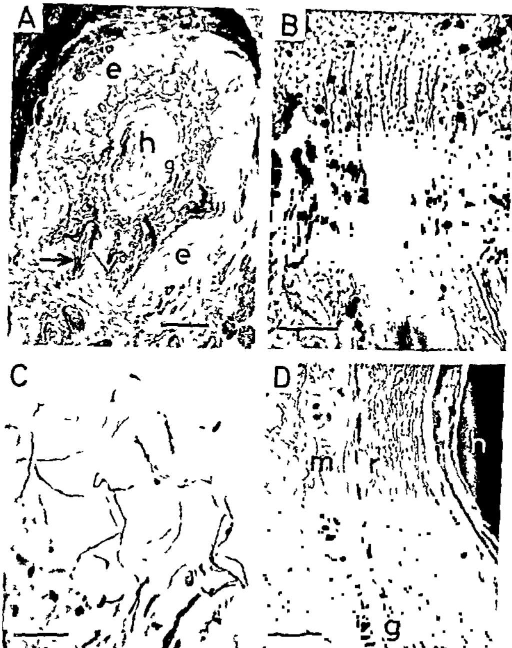


Fig 2 Structure of carpal sinus hair follicle

A cross section of follicle through lower cavernous portion of blood sinus At *arrow* a bundle of nerves penetrates the external sinus wall *e*=external sinus wall *g*=glassy membrane *h*—hair Black bar 100 μ

B oblique section through upper portion of follicle showing nerve fibers running parallel in the internal sinus wall Dark spots are artefacts caused by precipitation of silver grains Black bar 100 μ

C winding varicose fine nerve fibers in internal sinus wall in upper portion of follicle Black bar 30 μ

D cross section through upper portion of follicle showing nerve fibers in the internal mesenchymal sinus wall and nerve endings located close to Merkel cells in the external root sheath *m*=mesenchymal sheath constituting internal sinus wall *r* external root sheath *g*=glassy membrane *h* hair Black bar 30 μ (*A* *C* and *D* chloral hydrate silver nitrate method *B* Palmgren's method

Electrophysiological results

The first step in the analysis was to ascertain if the filament under study contained any fiber responding to mechanical stimulation of the skin. The type of afferent fiber thus found was identified by the responses obtained to displacements of sinus hairs, other hairs and the adjacent skin area, the movement being produced manually by means of an insulated probe under observation in a binocular dissection microscope. From these experiments it was evident that displacement of a carpal sinus hair could stimulate a number of different receptors of slowly as well as rapidly adapting type. In the present paper only responses from receptors in the sinus hair follicle will be analyzed; an account of other types of mechanoreceptors functionally associated with these sinus hairs will be given in the subsequent paper (Nilsson 1969).

A. Receptors adapting slowly to mechanical stimuli

Spontaneous activity. Many sinus hair units displayed a regular discharge of impulses in the absence of any intentional displacement of the hair. This activity, below designated spontaneous activity and observed in 17 of 29 single units, varied in frequency up to 20/sec.

Innervation pattern. A particular afferent unit could generally be activated only by displacement of a particular sinus hair but it might also be excited by displacement of an adjacent hair provided that the amplitude of the displacement of that hair was large enough. However, on a hair displacement of moderate amplitude a response was never obtained from more than one hair and it should thus be justifiable to conclude that each nerve fiber is associated with only one sinus hair. Each hair is, however, innervated by more than one fiber (*cf.* below).

Directional sensitivity. The sinus hair receptors displayed a certain degree of directional sensitivity insofar as displacement of a hair in one direction resulted in a strong discharge of impulses while movement in the opposite direction had no effect or might reduce the frequency of a spontaneous activity present. In order to classify each single unit on the basis of its directional sensitivity the hair was displaced manually in four directions at right angles to one another, *i.e.* proximally, distally, laterally and medially. As a rule, each unit was most sensitive to displacements in two of these directions. Out of 28 units tested, 12 were excited most effectively by proximal and lateral displacement. The next largest group consisted of 8 units responding most readily to proximal and medial displacement. Of the units activated by proximal displacement, two were equally sensitive to lateral and medial displacement. Only a few units (6 of the 28 tested) responded most effectively to distal displacement of the hair.

Conduction velocity. For 20 slowly adapting sinus hair units the conduction velocity in the afferent fiber was determined; the distribution of the values thus obtained appears from the histogram Fig. 3. Most of the fibers had a conduction velocity in the range 54—78 m/sec which, using a proportionality factor of 6 (Hursh 1939), would correspond to a fiber diameter between 9 and 13 μ.

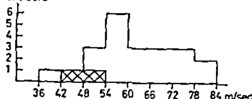


Fig 3 Histogram to show distribution of conduction velocities of 20 slowly adapting and 2 rapidly adapting (cross hatched) sinus hair units using class boundaries of 6 m/sec

Multiple innervation On several occasions two different units and on one occasion three units were found to be activated by displacement of one and the same sinus hair, the hairs were thus multiply innervated As a rule the individual units were identified in different filaments but in some cases displacements of a hair gave rise to impulse discharges in two fibers in one and the same filament as exemplified in Fig 4 These two units could be differentiated because the amplitude of the action potential of unit I was larger than that of unit II Unit I responded to medial (*A*) and unit II to lateral displacement (*B*) and both units were activated on proximal displacement (*C*) A sinus hair may thus be innervated by units of opposite directional sensitivity, this is an interesting functional mechanism which will be discussed below

Responses to displacements using the mechanical stimulator For the purpose of a quantitative analysis the hair was displaced with the mechanical stimulator and Fig 5 illustrates the response obtained in a sinus hair unit when the hair was moved at a constant rate to a new position and maintained at that position In this case the displacement rate was 14 4 mm/sec and the amplitude plateau 450 μ During the rising phase the response consists of a burst of impulses which changes into a discharge of lower frequency as soon as the plateau has been reached hence it is possible to split up the response into a dynamic and a static component In some cases these two components were separated by a brief silent interval but this was not a constant phenomenon Separate descriptions of each of the two types of response will be given below

Responses to static displacements Mechanical square wave pulses were used to study the receptor sensitivity to different positions of the hair After a short rise time

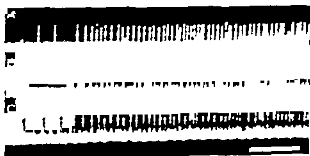


Fig 4 Responses of two different slowly adapting sinus hair units to displacement of same hair in different directions *A* unit I activated by medial displacement *B* unit II activated by lateral displacement *C* both units activated by proximal displacement of the hair Time bar 100 msec

Fig 5 Response obtained in slowly adapting sinus hair unit to movement of hair at a constant velocity of 14 4 mm/sec up to an amplitude plateau of 450 μ Time bar 100 msec

of 5—6 msec the plateau was attained Each experimental series comprised up to nine different displacement amplitudes Five stimuli were delivered at each amplitude at intervals of at least 12 sec and the repetitive stimulation was not found to affect the responses (*cf* Mountcastle *et al* 1966) Typical examples of these recordings are given in Fig 6, showing responses to static displacements of a hair at three different amplitudes, each about 1 sec in duration. After an initial discharge of a few impulses at short intervals in the brief dynamic phase a constant discharge is set up which after a rapid decline in frequency during the first 150—200 msec of the amplitude plateau displays a very slow adaptation The responses obtained in Fig 6 *A* and *C* to displacements of 150 and 500 μ, respectively are graphically represented in Fig 7, and a comparison of these responses reveals that the impulse frequency is related to the displacement amplitude A similar dependence on the displacement amplitude was observed also throughout more long lasting stimuli. After the first rapid decline, a phase of fairly constant frequency lasting 300—400 msec could be distinguished In analogy with similar phenomena observed in other types of slowly adapting cutaneous receptors this phase will be designated the early steady state (Werner and Mountcastle 1965) In order to determine the size of the static response at different positions of the hair the average frequency in this early steady state, *i.e.* usually 200—500 msec after onset of the displacement plateau was calculated, this value was reduced by the frequency of the spontaneous activity when present

The stimulus response relation for the static responses of the sinus hair receptors was analyzed by plotting the response (expressed in imp/sec) against the displace

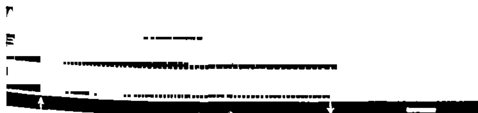


Fig 6 Responses obtained in slowly adapting sinus hair unit to static displacement of a hair at three different amplitudes each about 1 sec in duration Onset and end of stimuli at arrows *A* 150 *B* 325, and *C* 500 μ Time bar 100 msec (Note increase in postexcitatory pause with increasing stimulus intensity)

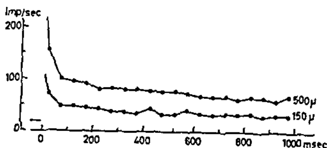


Fig 7

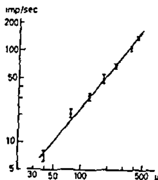


Fig 8

Fig 7 Graphical display of responses in the static phase of sinus hair displacements at two different amplitudes 150 and 500 μ corresponding to records *A* and *C* in Fig 6 Points show average impulse frequency calculated in time periods of 50 msec duration

Fig 8 Graphical representation on logarithmic coordinates of stimulus response relation of slowly adapting sinus hair unit on static stimulation at different amplitudes, five stimuli being delivered at each amplitude Responses expressed as average discharge frequency during early steady state after correction for spontaneous frequency Points represent mean frequency and lines range of observed values

ment amplitude (expressed in μ) on linear, semilog or log log coordinates There was then a good fit to a straight line in the case of the log-log representation over a wide amplitude range (*cf* Fig 8) which indicates that the static stimulus-response relation of these receptors can be described by a power function This power law relationship has been found to be valid also for other types of cutaneous mechanoreceptors in the cat (Werner and Mountcastle 1965, Janig *et al* 1968) and has the general form $R = k \cdot S^n$ where R is the size of the response, S is the size of the stimulus, and *k* is a proportionality factor The exponent *n* can be determined from the slope of the straight line in the logarithmic plot and when calculated for 15 units from 15 different hairs it was found to vary between 0 6 and 1 5 in individual units the range for eight of the units being 0 6—0 8 The value of the proportionality constant *k* varied in these units between 0 01 and 1 1

On stimulation at the maximal amplitude available, 450 μ, the discharge frequency of individual units in the early steady state differed over a range from 40 to 140 imp/sec However, stimulus amplitudes in different experiments should not be directly compared since the mechanical stimulus conditions may not be identical Thus for instance, it was technically difficult always to apply the probe at exactly the same position relative to the skin surface and to avoid minute variations in stimulus direction

Responses to dynamic displacements The dynamic responses were studied by recording the impulse discharge elicited by displacement of a sinus hair at varying velocities up to an amplitude plateau of 450 μ An experiment of this type is illustrated in Fig 9 showing responses to movements at six different velocities During

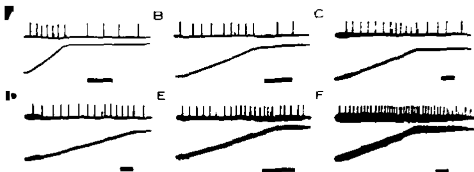


Fig 9 Responses of slowly adapting sinus hair unit to movement of hair at six different velocities up to a displacement amplitude of 450 μ. *A* 31 0, *B* 14 4, *C* 7 2, *D* 4 9, *E* 3 1, and *F* 1 4 mm/sec Time bar 10 msec in *A—D*, 50 msec in *E* and *F*

the fastest movement (*A*, 31 mm/sec) the response consists of a train of seven impulses at short intervals At the successively lower displacement rates in *B—F* (from 14 4 to 1 4 mm/sec, note different sweep velocities!) the duration of the rising phase increases, and hence a larger number of impulses are recruited in this phase, but it is also apparent from the records that the discharge frequency of the response is lower the slower the movement A closer study also revealed that at a certain rate of rise the impulse intervals are longest in the initial phase of the displacement and then gradually diminish These results are displayed graphically in Fig 10 which is a frequency-time plot of three of the responses (*B*, *C* and *E*) recorded in Fig 9 The three plots have some features in common As the movement starts there is a sudden stepwise increase in frequency from the spontaneous rate to a higher level The size of this step was related to the stimulus rate At the high rate of 14 4 mm/sec in *B*, the frequency rises to about 250 imp/sec while at the low rate of 1 4 mm/sec in *E* there is an initial increase in frequency only to 90 imp/sec In all these cases there was a gradual increase in frequency throughout the movement (*cf* above) Inspection of the responses obtained at different stimulus rates shows that the increase in frequency is relatively larger on slow movements During the final part of the movement the rise in frequency often diminished or the rate became constant As is also evident from the records in Fig 9 the maximal frequency attained in the final phase of the displacement was higher the faster the movement

Parallel with the sudden decrease in displacement velocity as the final amplitude was attained and the movement ceased there was also a decline in impulse frequency, the initial and major portion of which was very abrupt The later phase declined more slowly to a level approximately corresponding to that of the static response at this position of the hair As appears from the broken lines in Fig 10 the decline in discharge frequency when the hair movement ceased was of the same order as the stepwise increase at its initiation. This reduction in frequency is the effect on the receptor of a change in the velocity of the movement from a certain level to zero

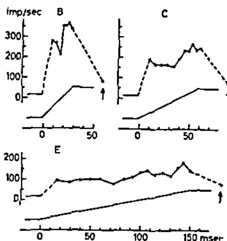


Fig 10

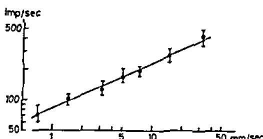


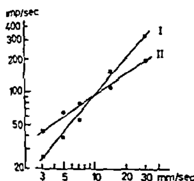
Fig 11

Fig 10 *Frequency time plot of three of the responses (B, C and E) recorded in Fig 9* Instantaneous frequency calculated as reciprocals of spike intervals and plotted along the time axis at the end of each interval Broken lines in the left part of the figure connect the level of spontaneous activity with the point representing the first impulse interval The right hand points, marked by arrow, show frequency of static response in the subsequent early steady state

Fig 11 Graphical representation on logarithmic coordinates of stimulus-response relation of slowly adapting sinus hair unit on dynamic stimulation at different velocities Responses expressed as increase in discharge frequency beyond the static response at the corresponding amplitude Dots give mean of five determinations at each velocity, lines indicating range of observed values

point during maintained displacement amplitude The size of this frequency change can thus be taken as a measure of the size of the dynamic response

As is evident from Fig 10, the size of the dynamic response is related to the displacement velocity, the receptors are thus velocity-sensitive To obtain a quantitative evaluation of the relation between displacement velocity and the size of the receptor response, the dynamic response was calculated as the difference between the average frequency in the final phase of the displacement (when the impulse intervals are fairly constant) and a value of the frequency in the subsequent static plateau phase As the latter value was chosen the frequency in the early steady state plateau period, in analogy with similar analyses performed on muscle spindles (Matthews 1963) A calculation of the response at different stimulus rates along these lines is displayed in Fig 11, in which impulse frequency has been plotted against displacement velocity on logarithmic coordinates five stimuli being delivered at each velocity The figure shows a good fit to a straight line When plotting the data on linear or lin-log coordinates on the other hand no straight line can be drawn through the points Analyses of the dynamic responses of eight other afferent units gave similar results, and hence it seems as though the relation between stimulus rate and dynamic response should be fully described by the power function mentioned above, *viz*: $R = k \cdot S^n$ where in this case R is the dynamic response in impulses per sec and S is the



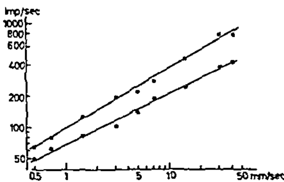


Fig 12 Fig 13

Fig 12 Comparison of stimulus-response relation of two slowly adapting sinus hair units (I and II) on dynamic stimulation Each point is the mean of five determinations

Fig 13 Stimulus response relation of slowly adapting sinus hair unit on dynamic stimulation *Squares* inward displacement, *dots* sideways movement

stimulus rate in mm/sec The value of the exponent n (the slope of the line) was calculated for nine units from seven different hairs and was found to vary between 0 5 and 1 1, for six of the units the exponent was lower than 0 9 The value of the proportionality constant k varied between 3 6 and 83

The differences in the dynamic responses obtained in different units are typically illustrated in Fig 12 In this experiment a filament was found to contain two units below designated I and II, which innervated two adjacent sinus hairs and whose directional sensitivity was of the same type Each of these two units was tested separately by dislocating the two hairs in succession in the same direction the probe being applied at equal distance from the skin surface In the figure the dynamic responses obtained in these units have been plotted against the displacement velocities The steeper slope of the line representing unit I (the value of n being 1 1 as against 0 6 for unit II) provides a quantitative measure of the higher dynamic sensitivity of this unit as compared to unit II

The sinus hair receptors were always very sensitive to pushing the hair in along its long axis The size of the dynamic response thus obtained at different velocities appears from Fig 13 (squares) At an amplitude of 250 μ the plateau was reached thus preventing the hair from being bent On inward displacements at the highest rates employed the impulse intervals recorded corresponded to a frequency between 900 and 1000 imp/sec which after correction for position response (cf above) corresponds to a dynamic response of close to 800 imp/sec On sideways movements at corresponding velocities the dynamic response never exceeded 500 imp/sec For comparison, Fig 13 also includes a plot of the stimulus response relation on sideways movements of the same hair (dots) In this case the movement reached the usual amplitude plateau of 450 μ As appears from the plots the stimulus response relations are similar for both types of stimulation The quantitative differences are

however of less significance since the mechanical conditions are quite different on inward and sideways displacements Thus for instance, in the latter case the hair acts as a lever and the actual displacement rate in the hair follicle is then lower than during a corresponding inward push, when the movement is more directly transmitted to the hair follicle

Thresholds Although the device used for recording of displacements did not permit accurate determinations of small amplitude differences, a rough estimate could be made of the thresholds for mechanical stimulation On sideways movements and when the probe was applied 2 mm above the skin surface the amplitude required for elicitation of an impulse discharge in the dynamic phase of the displacement using square wave pulses was 10—75 μ On pushing the hair inwards the threshold was usually below 10 μ

Effects of pressure changes in the blood sinus In an attempt to study if and to what extent the results might be influenced by mechanical events within the blood sinus, a small glass capillary with a tip diameter of 100 μ was inserted through the sinus wall This capillary was filled with a Ringer solution to which heparin had been added and the pressure in the interior of the sinus could be raised by injections of Ringer solution A rise in pressure was found to affect both the spontaneous activity when present and the responses to mechanical stimuli the impulse frequency was either increased or lowered and opposite effects might be observed in two units from the same hair However it proved to be difficult to achieve fully standardized experimental conditions since jet flows from the capillary tip in the course of the injections were apt to induce localized pressure effects and hair movements

Efferent stimulation Using fluorescence technique Fuxe and Nilsson (1965) could demonstrate the presence of adrenergic nerve terminals not only in the smooth pilomotor musculature around the sinus hair but also in the hair follicle This latter finding prompted an investigation designed to discover if the hair receptors might be influenced by an efferent intrasinal regulating mechanism Hence electrical impulses of different strengths and frequencies were delivered to the nerve supplying the sinus hair to see if they had any effects on the spontaneous activity and the responses to mechanical stimuli At low stimulus strengths approximately corresponding to threshold for the afferent fibers no definite effects were observed To activate the unmyelinized sympathetic fibers that must be postulated to be parent fibers of the adrenergic terminals observed in the follicle the electrical pulses were then increased to tenfold up to twentyfold strength and duration but then experimental difficulties arose since also pilomotor nerve fibers were activated At the stimulus frequencies used (1—10 c/s) a tetanic contraction in the sinus hair pilomotor musculature was induced (*cf* Nilsson 1968) The effect of this contraction on the sinus hair is that they stand up and move sideways in different directions and in this connection changes could be observed both in the spontaneous activity and in the responses to mechanical stimuli in some cases the discharge frequency increased in other cases it decreased on application of the electrical stimulation These changes may be due to the fact that the position of the hair relative to the

probe and hence the initial load were not the same It is also obvious that the hair receptors respond more readily to "physiological" stimuli on pilomotor contraction which results in erection of the hairs Whether the changes in discharge frequency observed may be attributable to some other factor than the movement of the hairs is difficult to decide, and no safe conclusions can yet be drawn as to whether or not there exists an efferent intrasinal mechanism directly affecting the behavior of the hair receptors

B Receptors adapting rapidly to mechanical stimuli

On movements of a carpal sinus hair also rapidly adapting impulse discharges may occasionally be recorded As will appear from the subsequent paper (Nilsson 1969) most of these responses can be shown to derive from activation of Pacinian corpuscles around the hair follicle, these receptors are recognized by their pronounced sensitivity to vibration If during the movement the sinus hair comes into contact with, or in some other way indirectly influences adjacent ordinary hairs rapidly adapting units from those hairs may also be activated

In some cases it has however been observed that a movement of a sinus hair may result in still another type of rapidly adapting responses That these responses do not derive from the types of receptors mentioned above could be established by vibratory tests and by closely clipping the ordinary hairs Hence it seems as though receptors of different degrees of adaptation should be present in the sinus hair follicle Since this observation should be of great basic interest, a short description of some of the characteristic features of these rapidly adapting units will be given

A few units of this type were studied more closely Like rapidly adapting receptors in general they were not spontaneously active On manual displacement of the sinus hair a train of impulses was generated during the movement but, by contrast to the previously described sinus hair receptors the discharges stopped as soon as the plateau was reached and the movement ceased Impulse discharges were elicited by dislocation in all directions also when the hair was pushed in toward the skin Pressure applied to the skin close to the sinus hair might give a response but in such cases this was likely to be due to an effect on the hair follicle In each single case this type of unit could be activated only by one sinus hair thus differing markedly from the rapidly adapting units associated with ordinary hairs which could always be activated by a number of hairs within a certain area (Brown and Iggo 1967)

Typical responses obtained in one of these afferent fibers when the sinus hair was displaced by means of the mechanical stimulator at three different velocities are displayed in Fig 14 The series of recordings shows that the response during the rising phase consisted of a burst of impulses whereas no discharges whatever were elicited during the plateau phase Occasionally some single impulses might however be set up at high displacement amplitudes Fig 14 *C* also shows the typical appearance of an off response as the hair resumes its normal position The responses to rapid movements (*A*—*B*) consisted of bursts of impulses at brief regular intervals On slow movements as in *C* the intervals were considerably longer, indicating

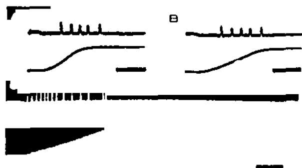


Fig 14 Responses of a rapidly adapting sinus hair unit to deflections of the hair at different rates up to an amplitude plateau of 450 μ *A* 63, *B* 42, and *C* 7 2 mm/sec Time bar 5 msec in *A* and *B*, 100 msec in *C*

that these receptors, like the slowly adapting ones, are velocity-sensitive During the first part of the movement in *C* the impulse intervals were fairly equal but in its later part they were more irregular and still longer In this respect these responses differ from those obtained from the slowly adapting sinus hair receptors, the frequency of which increased during the movement (*cf* Fig 9 and 10)

In contrast to the position-sensitive slowly adapting receptors, the rapidly adapting receptors were activated only on movements exceeding a certain minimum velocity In the case of one unit the critical slope was 2 3 mm/sec, while another unit responded to stimulation even at the lowest rate available with the stimulator employed (0 5 mm/sec) The irregularities in impulse intervals in the later part of the movement were most pronounced at velocities slightly above the critical slope

The conduction velocities of two fibers of this type (46 and 51 m/sec) have been included in the histogram in Fig 3, as seen, they are lower than those of most of the slowly adapting fibers This may indicate that the rapidly adapting units belong to another fiber population, but no definite conclusions can be drawn on the basis of the small number of data available so far

Discussion

Slowly adapting receptors In contrast to the ordinary covering hairs of the cat which have the double function of acting as a protective fur and of transmitting stimuli from the environment to the hair follicle receptors, the sinus hairs seem to have one specific function *viz* to transmit sensory information But so far only little is known about the purport of this information and its significance for the animal's behavior In previous investigations of these hairs attention has mainly been focused on the vibrissae which have been studied from various aspects In one of the few behavioral studies on this subject, Schmidberger (1932) showed that the locomotion is disturbed in blinded cats deprived of their vibrissae so that they fail to avoid objects in their way and have difficulties in walking along a narrow passage, whereas no such disturbances were observed in blinded cats with preserved vibrissae His experiments also suggested that the vibrissae may help the animal to "scan" its environment and to locate objects even before they come into contact with the vibrissae[1]

No similar experiments seem to have been performed to study sinus hairs on other parts of the body The possible function of the carpal hairs has given rise to many speculations based on the fact that these hairs are most common in animal species using their forelimbs to grasp their food or to feel and on Beddard s observations (1902) that some animals when holding their food place it in contact with the carpal hairs

The experiments reported in this and the immediately subsequent paper (Nilsson 1969) have demonstrated that the carpal sinus hairs represent an elaborate mechanism well suited to detect nearby movements and to provide tactile information about objects located in the vicinity of the paws

An extensive literature concerning the morphology of the vibrissae is available, thus *i a* two electron microscopic investigations have recently been published by Patrizi and Munger (1966) and by Andres (1966) The morphological findings reported in the present study established that there are no significant structural differences between the follicles of carpal sinus hairs and those of vibrissae even though the carpal hairs are clearly differentiated from the latter by the presence of Pacinian corpuscles and a smooth pilomotor musculature The studies of the innervation of the carpal sinus hairs demonstrated the presence both of free endings and of Merkel cell neurite complexes and also in this respect the carpal hairs resemble vibrissae (*cf* Andres 1966) In the rat and mouse vibrissae nerve fibers have been observed in the ringwulst (Vincent 1913 Melaragno and Montagna 1953) but no such fibers were seen in connection with the cat s carpal sinus hairs

The presence of nerve endings associated with Merkel cells is of interest since this receptor complex occurs in glabrous skin (Miller *et al* 1960) as well as in the hair discs and similar tactile pads in hairy skin (Pinkus 1905 Frankenhaeuser 1949 Iggo 196?) Mechanical stimulation of those receptors has been shown to elicit slowly adapting impulse discharges similar to the responses from receptors in the carpal sinus hair follicles recorded in the present study It is thus more likely that these latter responses are elicited in the Merkel cell neurite complexes present in the sinus hair root than in the free nerve endings

In the present series of experiments more than half of the carpal sinus hair units were spontaneously active and in this respect they differ from the afferent units of cat and dog vibrissae which were only occasionally found to be in a state of spontaneous activity (Fitzgerald 1940 Iggo 1968) In experiments of the type performed in the present study the proportion of spontaneously active fibers may however readily be overestimated since these fibers are more easily detected But since each single isolated filament was tested separately by movements of the sinus hairs only a fairly small number of fibers should have escaped notice Afferent units from tactile pads were either not spontaneously active (Tapper 1965 Iggo 1968) or discharged at very low frequencies (Werner and Mountcastle 1965) The absence of spontaneous activity is also characteristic of afferent units from the hair disc which is closely associated with the sinus hair and which will be described in the subsequent paper (Nilsson 1969) This difference with respect to spontaneous activi

observed in histologically apparently identical receptor complexes may be interpreted as being due to an actual functional dissimilarity, *e g* in the excitability of the nerve ending or to different mechanical conditions, *e g* in the initial load on the receptor This latter explanation gains some support from the fact that the activity may be abolished, at least transiently, by a dislocation of the hair (Nilsson and Skoglund 1965)

The analysis of a large number of afferent fibers from carpal sinus hairs shows that each fiber is associated with only one hair, and this seems to apply also to the afferent units of the vibrissae (Iggo 1968) This is in sharp contrast to the rapidly adapting units from ordinary hairs which can always be stimulated by movement of a large number of different hairs (*cf* Brown and Iggo 1967)

The receptors of the carpal sinus hairs are directionally sensitive and most of the units proved to respond most readily to proximal displacement Since the hairs are normally in a slightly oblique distal position the most effective stimulus is a movement erecting the hair, and this may be useful if the function of the hairs is to provide information for instance about a prey held between the forepaws In cross sections of the hair follicles the nerve fibers were not found to be distributed asymmetrically around the hair but this does of course not exclude that the terminals of a particular unit may be grouped at one side of the hair In his studies of the vibrissae, Fitzgerald (1940) found the directional sensitivity to be dependent on the position of the hair, the sensitivity of hairs near the mouth being of opposite direction as compared to hairs close to the lower edge of the orbit No such differential directional sensitivity could be observed for hairs located at different sites within the group of carpal sinus hairs in this respect these hairs thus behave as a functionally uniform group

When recording from multifiber filaments displacement of a hair in one direction could be seen to set up a discharge in one unit which however ceased firing when the hair was deflected in the opposite direction and was then substituted by another slowly adapting unit This multiple innervation of a hair by units of reciprocal responsiveness makes it possible to obtain more precise information about the hair movement than would be possible to deduce from a change in impulse frequency in a single fiber Changes in the position of the hair are also signaled by the rapidly adapting receptors as will be discussed below

The afferent fibers have the same conduction velocity as the most rapidly conducting cutaneous afferents of other types *e g* afferent units from tactile pads (Brown and Iggo 1967) a finding which is noteworthy in view of the assumption that the slowly adapting responses are initiated in Merkel cell neurite complexes in the sinus hair as well as in the tactile pad

The quantitative analysis of the responses of the slowly adapting receptors to displacement of sinus hairs shows that the behavior of these receptors is analogous to that of other types of slowly adapting mechanoreceptors Thus responses consisting of a velocity-dependent dynamic phase and a position-dependent static phase have been obtained from other types of cutaneous receptors such as tactile pad

receptors in the cat (Iggo 1968) and receptors in glabrous skin of the monkey's hand (Mountcastle *et al* 1966) as well as from muscle spindles (Katz 1950 Ottoson and Shepherd 1965)

The responses of the sinus hair receptors to static displacement displayed a brief transient phase of declining impulse frequency levelling off to a fairly constant discharge rate The quantitative analysis of the static responses was based on the average impulse frequency in this early steady state and showed that the frequency was related to the displacement amplitude by a power function A similar stimulus response relation has been found to exist for the cat's tactile pad receptors (Werner and Mountcastle 1965) and for other slowly adapting cutaneous receptors of various types (Janig *et al* 1968 Siminoff 1968) whereas *e g* monkey hand receptors have been shown to display a linear stimulus response relation (Mountcastle *et al* 1966)

The responses to static displacements were somewhat varying in different sinus hair units as mirrored by the variations in the exponent *n* of the power function even though values both above and below 1 were encountered the values found for most of the units ranged between 0 6 and 0 8 As mentioned above no direct comparisons with other receptors can be made in view of the differences in mechanical stimulus conditions but it is interesting to note that both Werner and Mountcastle (1965) and Janig *et al* (1968) have found that the values of *n* were commonly less than 1

In the dynamic phase a discharge of gradually increasing frequency was set up in the slowly adapting sinus hair receptors and the final frequency attained was considerably higher than that of a static response at the same hair position This clearly illustrates the pronounced dynamic sensitivity of these receptors The analysis of the dynamic response defined as the increase in frequency beyond the position response (*cf* Matthews 1963) revealed that it is related to the velocity of the movement by a power function In studies of the dynamic responses from tactile pad receptors in the cat, Tapper (1965) found this power law to be valid for the relation between frequency in the rising stimulus phase and displacement velocity

In experiments on isolated frog muscle spindles Shepherd and Ottoson (1965) showed that there was a close fit to a straight line in a log log diagram for the relation between the final frequency of a dynamic impulse discharge and the velocity of stretch In a subsequent paper (Ottoson and Shepherd 1965) they were able to correlate this to the finding that a similar relation exists between the rate of stretch of the spindle on the one hand and the rate of rise and the dynamic peak amplitude of the generator potential on the other hand It would be of interest to know the corresponding potential changes occurring in the sinus hair receptors but attempts made to record a generator potential by means of an electrode inserted into the sinus of a hair were not successful

In their capacity to respond to changes both in position and velocity the sinus hair receptors differ from the rapidly adapting receptors of the ordinary hairs which are exclusively velocity sensitive In studies of the dynamic responses of these latter receptors Brown and Iggo (1967) found that also in these cases the stimulus

response relation can be described by a power function The frequency of the dynamic response of the ordinary hairs at different stimulus rates was of about the same order as the dynamic response of the sinus hair receptors at corresponding velocities

According to Iggo (1968) each tactile pad contains 30—50 Merkel cells The number of Merkel cells in the sinus hair follicle is not known but each afferent sinus hair unit is probably associated with several such cells It is thus likely that the impulse discharges in a sinus hair unit may be influenced from several Merkel cell neurite complexes in the same manner as activity at different receptor sites within a tactile unit in toad skin is integrated (Lindblom 1958) Very little is known about the mechanical events in the hair follicle but on histological preparations of vibrissal follicles Wrobel (1965) has shown that curvings of the hair root may result on deflection of the hair Such curvings cause a change in the shape of the cells in the root sheath in that they become flattened on the convex side and more rounded on the concave side Also in the carpal sinus hairs nerve impulses may be induced by such pressures and tensions The site of this cell deformation will be dependent on the direction of the hair deflection and an asymmetrical arrangement of the Merkel cell neurite complexes of each afferent unit may explain the difference in directional sensitivity observed in units from one and the same sinus hair

Rapidly adapting receptors The recording of rapidly adapting responses from receptors in the sinus hair follicle is of interest from a functional aspect insofar as these responses add to the information about hair movements provided by the slowly adapting receptors Since the rapidly adapting receptors have no directional sensitivity they respond also when the hair resumes its normal position thus increasing the effect of the reciprocal directional responsiveness discussed above The significance of the joint information thus reaching the central nervous system from different receptor types in and around the hair follicle will be more extensively discussed in the subsequent paper (Nilsson 1969)

Fitzgerald (1940) found the nerve endings of the vibrissae to be mainly slowly adapting Sometimes also rapidly adapting discharges were recorded in single fibers from the region of the vibrissae but in most cases such discharges seemed to derive from fine hairs surrounding the vibrissae The possibility that some of the endings of the vibrissae are rapidly adapting was however pointed out

The rapidly adapting responses from the carpal sinus hairs resembled the responses from common hair receptors (Brown and Iggo 1967) in all respects except insofar as they could only be elicited from a single hair A prerequisite for their activation was thus a certain minimum velocity and the frequency of the response was rate-dependent Also responses from common hairs displayed more regular impulse intervals at higher stimulus velocities

As described above the sinus hair follicles were found to contain in addition to probably slowly adapting Merkel cell neurite complexes also free nerve endings which might be able to set up rapidly adapting responses In that case the adaptation differences observed might be due to differences in the receptor properties But

there is also the possibility that mechanical conditions may play an important part in the degree of adaptation Thus the Merkel cell neurite complexes are located inside the strong glassy membrane in the external root sheath, which is composed of a dense tissue capable of effectively transmitting and sustaining mechanical tensions whereas free nerve endings have mainly been observed in the relatively soft mesenchymal interior sinus wall

Some additional comments In a previous paper (Nilsson and Skoglund 1965) certain hypotheses concerning the functional significance of the blood sinus were reviewed The significance of the mechanical conditions has been extensively discussed by Wrobel (1965) In the present paper some experiments have been reported suggesting that the receptor response may be influenced by pressure variations within the sinus

The finding of an adrenergic innervation of the sinus hairs (Fuxe and Nilsson 1965) raised the question if the sensitivity of the sinus hair receptors may be governed by some mechanism similar to that influencing the receptors in frog skin by activity in sympathetic nerves (Loewenstein 1956) However it proved to be necessary to abstain from a further study of this problem since in experiments with stimulation of sympathetic fibers it was difficult to distinguish a possible effect of the type suggested above from an effect of the concomitant contraction in the pilomotor musculature But it is evident that the pilomotor contraction may constitute a component in an efferent mechanism by inducing a hair erection thus making the hairs more accessible to mechanical stimuli

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Hair Discs and Pacinian Corpuscles Functionally Associated with the Carpal Tactile Hairs in the Cat

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Abstract

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A hair disc containing Merkel cell neurite complexes at the base of the epidermis lateral to each carpal sinus hair in the cat is demonstrated The physiological properties of these receptors and of the Pacinian corpuscles around the sinus hair root were analyzed using electrophysiological techniques and a mechanical stimulator permitting displacements at different rates and amplitudes The hair disc receptor could be excited by direct mechanical stimuli and by lateral deflection of the hair The dynamic response was displacement rate sensitive The slowly adapting static response was related to the displacement amplitude The Pacinian corpuscles followed a sinusoidal skin stimulation from 20 *c/s* to 300—400 *c/s* A plot of the lowest amplitude giving one imp/cycle at different frequencies gave a L shaped curve with lowest threshold at 125—250 *c/s* These receptors were also sensitive to vibratory stimulation of the hairs on rapid hair displacement on and off responses resulted An example is given of simultaneous activation of slowly adapting hair receptors and Pacinian corpuscles by displacement of a hair The functional significance of the unique combination of four different types of mechanoreceptor in "the tactile hair organ" is discussed

On mechanical stimulation of the carpal sinus hairs on the cat's forelimbs and the surrounding skin area both rapidly and slowly adapting impulse discharges are elicited in the cutaneous nerve from this area and one type of rapidly adapting responses has been shown to derive from vibration sensitive Pacinian corpuscles around the blood sinus of the hair follicles (Nilsson and Skoglund 1965) The responses set up in receptors in the sinus hair follicle were extensively analyzed in an immediately preceding paper (Nilsson 1969)

Continued analysis of the impulse discharges in this cutaneous nerve revealed that slowly adapting responses can also be obtained on touch of a particular spot of the skin close to each sinus hair In the investigation reported in the present paper the receptors from which this type of slowly adapting responses originates have been identified histologically as Merkel cell neurite complexes present in a hair disc and their physiological properties have been studied

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The paper also provides a quantitative analysis of the excitability properties of the Pacinian corpuscles around the sinus hair

A series of experiments will be presented showing that each of these two receptor types is activated together with the hair follicle receptors on mechanical stimulation of the sinus hair, in virtue of their close anatomical contact with the hair, and different aspects of the functional significance of this triad of receptor types will be discussed

Methods

Experiments were carried out on fifteen cats which were anesthetized with Nembutal 40 mg/kg bw, given intraperitoneally The technique employed to record the impulse activity in single afferent units as well as the mechanical stimulator used to achieve precise control of hair and skin displacements were described in detail in the preceding paper (Nilsson 1969) For vibratory stimulation of Pacinian corpuscles the stimulator was driven by sine waves from an audio-frequency generator The conduction velocity in the afferent fibers was determined by means of the collision method developed by Brown and Iggo (1967) and modified as described in the preceding paper

The sinus hairs with surround
fixed in formalin and stained with
the preparations were either fi
according to Palmgren's method

Results

A The hair disc

Morphology When studying the morphological features of the skin surrounding the carpal sinus hairs under a binocular dissection microscope the blood sinus of the hair follicles can be clearly discerned as a purple blue, slightly elevated ring enclosing each sinus hair In this skin area there are no ordinary hairs

A lateral portion of this ring approximately one third of its circumference is slightly more elevated than the surrounding tissue and pink in color Histological sections reveal that the epidermis in this area is composed of a larger number of cell layers than normal and about twice as thick as in the surrounding skin From Fig 1 *A*, showing a section containing three sinus hair follicles this thickened portion of the epithelium can be seen (at arrows) to be situated in the immediate vicinity of and lateral to the mouths of the follicles and at the hair to the far left in the figure it can be seen extending into the follicle mouth Its close connection with the sinus hair is also evident from Fig 1 *B* in which this epithelial structure can also be seen to be distinctly separated from the surrounding tissue In nerve stained sections nerve fibers can be seen ascending from the deeper parts of the skin and branching beneath this specific portion of the epidermis (Fig 1 *C*) At the boundary between the epidermis and the corium there is a row of large pale cells with a lobulated nucleus i.e. typical Merkel cells (Fig 1 *D*) In single sections fine nerve terminal can be seen close to these cells The subepidermal connective tissue is richly vascularized hence the pink color of the skin In its structure this organ bears a striking resemblance to the hair disc (Haarscheibe) first described by Pinkus (1902), and in the following description of the organ the term hair disc will be used

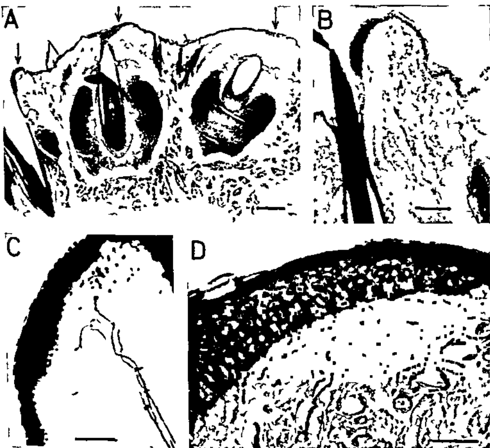


Fig 1 Structure of hair disc of carpal sinus hairs
A section through group of sinus hairs demonstrating three follicles *Arrows* indicate sites of hair discs which appear as a thickening of the epidermis to the right of (lateral to) each follicle mouth Black bar 300 μ
B section through the mouth of a sinus hair follicle showing close connection between hair and hair disc Black bar 100 μ
C nerve fibers branching below hair disc Black bar 50 μ
D a row of pale large Merkel cells at the base of the hair disc Beneath the epidermis nerve fibers can be seen to the left and blood vessels to the right Black bar 25 μ
A and *D* chloral hydrate silver nitrate method *B* and *C* Palmgren s method

A study of the area by means of Falck and Hillarp s fluorescence method revealed that the Merkel cells are devoid of catecholamines (Fuxe and Nilsson unpublished observations)

Physiology Once a small nerve filament had been dissected free and lifted onto the recording electrode the first procedure was to check if it contained any unit responding to mechanical stimulation of the skin area containing the sinus hairs In some cases an afferent unit responding to touch of a small spot lateral to a sinus hair could then be identified On manual application of pressure to the skin

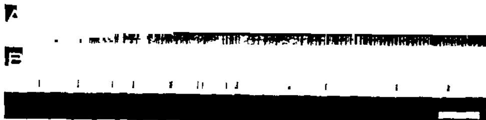


Fig 2 *A*, slight manual pressure applied to hair disc induces slowly adapting response *B* same unit responding to lateral deflection of the associated sinus hair Time bar 100 msec

with a small probe under microscopic observation the sensitive spot was found to be identical with the pink elevation close to the sinus hair described above, *viz* the hair disc No response was induced by slight touch of the skin close to the disc or by pressure applied to hair discs close to adjacent sinus hairs In some cases the most sensitive spot was located inside the follicle mouth (*cf* the hair follicle to the far left in Fig 1 *A*) One and the same hair disc was never supplied by more than one afferent unit

No spontaneous activity was present in the hair disc units A typical impulse discharge in response to mechanical stimulation is illustrated in Fig 2 Manual pressure applied to the hair disc with the probe gave a slowly adapting response (*A*) of fairly high frequency, in this case about 350/sec The same unit also responded (*B*) to lateral deflection of the sinus hair *viz* towards the hair disc (*cf* Fig 1 *A* and *B*) but a medial hair movement did not evoke any response in these receptors Nor did the hair disc unit respond when the hair was pushed in toward the skin a movement which invariably activates the slowly adapting sinus hair units (Nilsson 1969) This is apparent from the records in Fig 3 from an experiment in which a single filament was found to contain a fiber from a sinus hair as well as also a fiber from the hair disc associated with this hair In *A* slight touch of the hair disc produced a response in *B*, inward pressure of the hair did instead give a response in the sinus hair unit the spikes of which were of higher amplitude The different responses thus obtained made it possible with full certainty to distinguish between these two types of slowly adapting units

The adaptation properties of the hair disc receptors were studied using displacements of different amplitudes The mechanical stimulator was moved downwards until the tip of the probe made contact with the hair disc perpendicular to the skin



Fig 3 Recording from filament containing a fiber from a sinus hair and another fiber from its hair disc
A hair disc unit activated by slight manual pressure applied to the hair disc
B sinus hair unit activated by pushing the hair in toward the skin
Time bar 100 msec

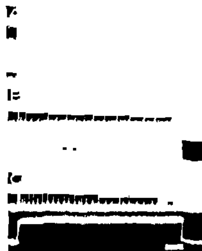


Fig 4 Responses of hair disc unit to static displacements of hair disc of different amplitudes *A* 38 *B* 175, and *C* 237 μ Time bar 100 msec

surface In the experiment illustrated in Fig 4 the hair disc was displaced to different amplitude plateaus At the lowest amplitude (*A* only a single impulse discharge was set up in the dynamic phase while the stimulus was subthreshold for the static response On stimulation at higher amplitudes (*B* and *C* respectively) a continuous impulse flow was maintained throughout the plateau phase During the first 200 msec of this phase the discharge declined in frequency and then changed into a comparatively constant phase The frequency in the later steady state phase was dependent on the stimulus amplitude No extensive quantitative analysis of the static response was made since the results obtained agree well with those reported in previous studies of other receptors of this kind not associated with sinus hairs (Iggo 1962, Werner and Mountcastle 1965) The hair disc of the carpal sinus hair is also less suitable for such analyses since it is difficult to achieve constant stimulus conditions, the hair disc being apt to slip off the probe For the same reason no quantitative analysis of the dynamic response was made

In some experiments however the hair disc was activated by displacements at various velocities in analogy with the experiments reported in the preceding study of the sinus hair receptors (Nilsson 1969) in which it was shown that the impulse frequency was related to the rate of stimulation Also the hair disc receptors were found to be velocity dependent i.e. the faster the movement the higher the frequency of the discharge (cf Tapper 1965) Fig 5 shows recordings from an experiment of this type at different displacement rates in which during the fastest movement (*A* 31 mm/sec) impulses were recruited at short intervals corresponding to a frequency of 375/sec while at the slowest movement (*F* 1 4 mm/sec) the impulse intervals corresponded to a frequency of only 60/sec The impulse intervals were either fairly uniform throughout the movement as in Fig 5 or briefer in its later phase

The conduction velocity as measured in five fibers of this type ranged from 56 to 70 m/sec

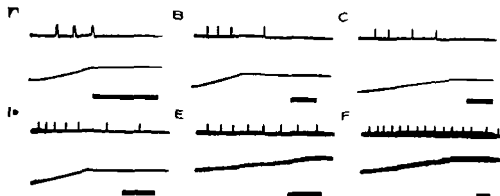


Fig 5 Responses of hair disc unit to displacements of hair disc at different velocities up to a displacement amplitude of 237 μ *A* 31 0, *B* 14 0, *C* 7 2, *D* 3 1, *E* 1 4, and *F* 0 7 mm/sec Time bar 10 msec in *A—C*, 50 msec in *D—F*

B The Pacinian corpuscles

The Pacinian corpuscles around the carpal sinus hairs are exquisitely sensitive to, *i e*, a slight tap on the experimental table and to a vibrating probe applied close to bony structures in the lower leg, in this respect they resemble the corpuscles located more deeply in the lower leg (Skoglund 1960) On application of a sinusoidal vibratory stimulus the stimulus threshold was lowest when the probe was in contact with the skin close to the carpal sinus hairs and when it touched the proximal part of a sinus hair Farther up on the hair the threshold was, as expected slightly higher and of the same order as on vibratory stimulation of the skin 1—2 cm from the sinus hair group All the sinus hairs could activate a particular vibration-sensitive unit, and there was no appreciable threshold difference when vibration was applied to different hairs

The response to sinusoidal stimulation with the probe in contact with the skin close to the sinus hairs is illustrated in Fig 6 At frequencies of 75 and 100 c/s (*A* and *B* respectively) one or two impulses were recruited in each stimulus cycle At frequencies exceeding 100 c/s (*C* and *D*) the response consisted of a regular dis-

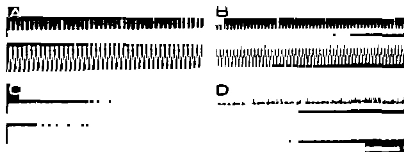


Fig 6 Responses of Pacinian corpuscles to sinusoidal stimulation of the skin close to the sinus hairs *A* 75 *B* 100 *C* 200 and *D* 250 c s Time bar 100 msec

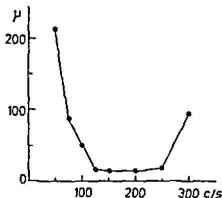


Fig 7 Threshold frequency curve obtained on sinusoidal stimulation of Pacinian corpuscle *Abscissa* frequency in *c/s*, *ordinate* threshold stimulus amplitude giving one impulse per cycle

charge of one impulse per cycle This unit followed a sinusoidal stimulus responding with at least one impulse per cycle from 22 c/s up to a maximum frequency ranging between 300 and 400 c/s The lowest amplitude resulting in at least one impulse per cycle was measured at different frequencies, as exemplified in Fig 7 The course of the curve is U-shaped, the threshold being lowest in the range between 125 and 250 c/s and higher on either side of this minimum These receptors were also tested by rapid sideways deflections of a sinus hair, and the response thus obtained appears from Fig 8, in which the probe was placed in contact with a sinus hair 2 mm from the skin surface On movement at different velocities the critical slope was found to be 60 mm/sec In another experiment in which the probe was applied in contact with the skin the critical slope was 10 mm/sec Fig 8 also shows that a response was also elicited at the end of the plateau phase when the hair resumed its normal position On manual stimulation this off response could easily be demonstrated by moving the sinus hairs sideways and then suddenly releasing them

In recordings from multifiber filaments it could be clearly seen how a rapid movement of a sinus hair resulted in a simultaneous activation both of Pacinian corpuscles and of receptors in the hair follicle In the experiment Fig 9 *A* a rapid manual deflection of a sinus hair sets up a brief train of impulses from Pacinian corpuscles (large diphasic spikes) and results in an increase in frequency of the pre existent discharge of a hair follicle unit (small spikes) When the hair was then relatively slowly brought back to resting position, no responses were obtained from the Pacinian corpuscles (Fig 9 *B*) A silent period in the discharge of small spikes can



Fig 8 On and off responses of Pacinian corpuscle induced by rapid deflection of sinus hair Time bar 10 msec



Fig 9 Deflection of sinus hair inducing responses in three different fibers ii one from a Pacinian corpuscle (large spikes) and two from the follicle of the sinus hair (small and intermediate spikes) *A* onset of lateral deflection (indicated by white line) *B*, at the end of this deflection fairly slow return of hair to normal position *C*, first part of slow medial deflection *D*, at the end of this deflection rapid return of hair to normal position Full description in text Time bar 100 msec

be seen at the end of the stimulation and at the same time a few spikes of intermediate amplitude are set up Deflection of the hair in the opposite direction (Fig 9 *C*) revealed that these latter spikes derived from a sinus hair unit of opposite directional sensitivity On this movement a transient 'inhibition of the spontaneous discharge of the small amplitude unit occurred The hair movement in *C* was too slow to excite Pacinian corpuscles but when the hair was rapidly released (*D*) they responded with a brief burst of impulses

The conduction velocity as determined in three fibers from Pacinian corpuscles ranged between 58 and 67 m/sec

Discussion

The results reported in this and an immediately preceding paper (Nilsson 1969) have demonstrated that the carpal sinus hairs constitute the central component of a unique receptor complex comprising (1) hair follicle receptors mainly of slowly but also of rapidly adapting type (2) a hair disc intimately associated with each single hair and (3) Pacinian corpuscles located more deeply around the hair follicles In the following the individual properties of these two latter receptors as well as some aspects of the integration of the different receptor types will be considered

Hair discs associated with carpal sinus hairs have not been described previously nor are any hair discs mentioned in the extensive early literature on the vibrissae but in a recent publication Mann (1968) has demonstrated the presence of hair discs associated with vibrissae in the American opossum Hairs discs are however generally present in hairy skin either in connection with tylotrich hairs (Pinkus 1902 1905 Straile 1960) or as independent epidermal pads in the latter case they are often called touch spots touch corpuscles or tactile pads (Frankenhaeuser 1949 Iggo 1962 Lindblom and Tapper 1967) In their microscopical structure the hair discs of the carpal sinus hairs do not differ significantly from the corresponding epidermal pads described at other sites (*cf* Smith 1967 Iggo 1968)

The hair discs of the carpal sinus hairs are exquisitely sensitive to mechanical stimuli which set up both a velocity-dependent dynamic response and an amplitude dependent slowly adapting static response, in this respect they resemble the corresponding receptors in hairy skin (Werner and Mountcastle 1965 Tapper 1965 Iggo 1968) The mechanoreceptive part of the hair disc is held to be the Merkel cell neurite complexes at the base of the epidermis (*cf* Iggo 1968) The carpal sinus hair discs are not multiply innervated and also in this respect they resemble the hair discs in hairy skin However, whereas in hairy skin up to seven hair discs have been identified as being associated with one and the same afferent fiber (Tapper 1965 Brown and Iggo 1967) the present investigation revealed that only one carpal sinus hair disc is associated with each afferent nerve fiber

The physiological studies of Pacinian corpuscles in the extremities performed up to this time have mainly been concerned with corpuscles located beneath glabrous skin (Gray and Matthews 1951 Lindblom and Lund 1966 Janig *et al* 1968) and close to deeper structures such as fasciae and tendons (Skoglund 1960 Hunt and McIntyre 1960) as well as joint capsules (Boyd 1954) But it seems to be uncommon that Pacinian corpuscles occur superficially in hairy skin and hence the comparatively large number of such corpuscles found close to the carpal sinus hairs is all the more remarkable Even though these corpuscles are located in a relatively soft tissue—which may seem to be a less appropriate site for a receptor of this type—they respond effectively not only to vibration applied to the sinus hair but also to more distant vibratory stimuli The U shaped threshold frequency curve obtained on sinusoidal stimulation agrees well with similar curves from previous investigations on isolated Pacinian corpuscles (Sato 1961) and on Pacinian corpuscles in monkey and cat foot (Lindblom and Lund 1966 Janig *et al* 1968) Also in these earlier investigations a minimum frequency of about 20 c/s was observed below which the receptors did not follow a sinusoidal stimulation Hunt and McIntyre (1960) and Hunt (1961) found a somewhat higher limit 40 c/s for Pacinian corpuscles in the cat s hind limb Those receptors could follow a vibratory stimulus up to a frequency of 600 c/s whereas the upper boundary observed in the present work ranged between 300 and 400 c/s These values as also the critical slope on stimulation applied to the skin close to the sinus hairs are however consistent with the values found for Pacinian corpuscles in monkey foot (Lindblom and Lund 1966) The higher velocity of displacement required to excite a unit on deflection of the sinus hair may be due to the fact that the hair acts as a lever and that thus the actual velocity in the hair root is lower But whatever type of stimulus was applied the critical slopes as measured in the present experiments were higher than those observed for rapidly adapting receptors in the sinus hair follicle (Nilsson 1969) and in ordinary hair follicles (Brown and Iggo 1967)

Pacinian corpuscles frequently occur close to blood vessels both in cat mesentery (Gammon and Bronk 1935) and in human digits (Cauna and Mannan 1958) Even though pulse synchronous discharges have occasionally been recorded from Pacinian corpuscles located around the carpal sinus hairs (Nilsson and Skoglund 1965) it is

still an open question whether these corpuscles normally react to the blood flow in the sinus

The carpal sinus hairs are better suited than the ordinary hairs to provide tactile information since they are longer, thus projecting above the rest of the coat and thicker and stiffer and hence more effective in transmitting mechanical stimuli to the hair follicle When these hairs are deflected by contact with an object not only nerve endings in the hair follicle and in the hair disc but also Pacinian corpuscles can be activated On lateral deflection of a sinus hair *i.e.* toward the hair disc the disc receptors are excited but on medial deflection or when the hair is pushed in toward the skin sinus hair units are excited without any co activation of the hair disc (*cf* Fig 2 and 3) Since the hair disc of the carpal sinus hairs is always located lateral to the hair the directional information thus obtained can add to the information mediated through the directionally sensitive slowly adapting hair follicle receptors (Nilsson 1969) The position of the tylotrich hair relative to the hair disc may not invariably be the same (Smith 1967) and consequently this hair disc is not capable of transmitting directional information even though it may be excited by a movement of the tylotrich hair (Brown and Iggo 1967)

If on mechanical stimulation some vibratory component is involved this is primarily picked up by the Pacinian corpuscles since the hair follicle receptors have no appreciable vibratory sensitivity Under normal physiological conditions such a vibratory component may be initiated either by direct contact between the hair and a vibrating object or by air currents set up by a moving object Owing to technical difficulties the responsiveness of the hair disc receptors to vibratory stimuli was not tested in the present experiments but if these receptors respond as effectively to a sinusoidal stimulus as does the same type of receptor in hairy skin (Lindblom and Tapper 1967) they may in some cases transmit useful information about vibratory stimuli

By the activation of a number of different receptor types on deflection of a carpal sinus hair a larger amount of information about the movement of the hair can be transmitted to the central nervous system thus providing a more distinct perception of the hair movement Thus for instance on lateral dislocation of a hair the onset of the movement is signaled by discharges from Pacinian corpuscles while both rapidly and slowly adapting hair follicle receptors as well as the hair disc receptors give information about the velocity of the movement The two latter slowly adapting receptors are also dependent on the displacement amplitude and thus transmit information about the degree of the deflection The mechanical pulses employed in the analysis of the receptor properties in the present experiments are no doubt smoother than normal physiological stimuli which probably consist in small displacements and vibrations added to a larger more or less static dislocation In view of the combination of a number of receptors possessing different properties the carpal tactile hair organ provides a material well suited for analyses of such complex stimuli

A functional coupling of *two* mechanoreceptors possessing different properties is

not an uncommon arrangement. Thus the tylotrich hair constitutes a receptor complex composed of a rapidly adapting hair follicle receptor and a slowly adapting hair disc receptor. Pressure applied to scales in alligator skin sets up responses both in rapidly and slowly adapting afferent units (Siminoff and Kruger 1968). The tactile hairs of insects are of two types, one responding to steady deformation and another reacting only during movements (Wolbarsht and Dethier 1958). Similar combinations can be observed also in non-cutaneous receptors. Thus for instance movement of a joint may set up slowly adapting responses from Ruffini endings and rapidly adapting responses from Pacinian-like receptors (Boyd 1954). The mammalian muscle spindle consists of two slowly adapting components of different dynamic sensitivity (cf. Matthews 1963), whereas the crayfish muscle stretch receptor is composed of two cells, one responding to stretch by rapidly adapting, another by slowly adapting impulse discharges (Eyzaguirre and Kuffler 1955).

Even though it is evident from these examples that a combination of receptors is fairly common, a close association of more than two types of receptor is uncommon and in this respect the carpal tactile hair organ seems to be a unique functionally integrated structure, since a mechanical stimulus applied to a single tactile sinus hair may simultaneously activate up to four different receptor types.

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Renal Function in Man during Transition from Hydropenia to Water Diuresis with Superimposed Mannitol Load

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Abstract

Aperia, A., O. Broberger, N. O. Ericsson and H. Feychting. *Renal function in man during transition from hydropenia to water diuresis with superimposed mannitol load.* Acta physiol. scand. 1969. 77. 429—438.

Renal function studies have been carried out in seven healthy volunteers during the transition from hydropenia to water diuresis with superimposed mannitol load. The glomerular filtration rate increased significantly during the transition from hydropenia to water diuresis but re

clearance were corrected for. Those results were interpreted to mean an increased water permeability in distal tubule of the human nephron during hydropenia.

Micropuncture studies of tubular fluid in dog and rat have proved the existence of the countercurrent system and the importance of active Na reabsorption for concentration and dilution of the urine. Studies on the urinary excretion of Na and other solutes, free water reabsorption and free water formation under various diuretic states have indicated that urine concentrating and diluting mechanisms are the same in man (Goldberg *et al.* 1964, 1965, Steinmetz *et al.* 1964).

Recently certain species differences between dog and rat have been demonstrated. Micropuncture studies have shown that during hydropenia the osmolality of late distal tubular fluid in rat is significantly higher than during water diuresis (Gottschalk and Mylle 1959). In contrast to this the late distal tubular fluid in dog is almost equally hypotonic during hydropenia and water diuresis (Clapp and Ro

TABLE I L W, male, 22 years

Time	V ml/min	GFR* ml/min	C_{PAH}* ml/min	Cosm ml/min	$T^c_{H_2O}$ ml/min
—80— 40	0 82	99	456	2 1	1 31
—40— 0	0 89	88	476	2 2	1 27
0— 50		Drinks 1500 ml water, continued water intake slightly			
50— 60	14 0	103	547	4 4	
60— 70	14 9	105	566	4 7	
70— 80	16 0	112	531	4 5	
80— 90	17 2	109	667	4 6	
90—100	17 4	106	564	4 4	
100		Infusion of 3 % mannitol solution started			
100—110	18 6	110	575	5 8	
110—120	23 3	97	527	8 0	
120—130	26 3	103	620	10 5	
130—140	26 7	97	643	11 1	
140—150	29 7	96	657	13 0	
150—160	29 3	101	649	12 9	
160—170	29 5	104	635	13 7	

* GFR and C_{PAH} calculated per 1 73 m² body surface

son 1966) For obvious reasons micropuncture samples have not been obtained from the human kidney The present report is an indirect attempt to study the distal tubular reabsorption of Na^+ and water during hydropenia and water diuresis in normal man For this purpose each individual was studied during the transition from hydropenia to water diuresis and in most cases also from water diuresis to water diuresis with superimposed mannitol load This procedure allowed comparisons between actual and expected dilution of urine and excretion of osmotic load and Na^+ during various diuretic states without regard to nutritional state or electrolyte homeostasis Evidence will be presented for an increased water permeability in the distal tubule of the human nephron during hydropenia

Materials and Methods

[illegible] ned from [illegible] rs For [illegible] pplied [illegible] ived a [illegible] a rate [illegible] tarted [illegible]

Studies in the hydropenic state comprised 2— [illegible] 30 min [illegible] samples were drawn in the middle of each period from an indwelling Stille Werner cannula in a peripheral vein

After completion of the hydropenia studies water diuresis was induced by allowing the volunteers to drink water in an amount of 2 per cent of the body weight initially and thereafter 0 5 per cent of the body weight every 30 min Water diuresis was believed to be complete when

C_{H_2O} ml/min	$U_{Na}V$ µeq/min	C_{Na}/C_{In} %	U_KV µeq/min	U_{osm} mO/l	Serum Na meq/l
	165	1 15	75	820	145
	171	1 33	69	790	145
exceeding the diuresis					
9 5	307	2 08	61	105	143
10 2	283	1 89	58	100	142
11 5	271	1 73	55	93	140
12 6	241	1 58	58	85	139
13 0	244	1 63	59	85	140
12 9	242	1 60	54	98	138
15 3	327	2 47	49	114	137
15 8	447	3 19	47	127	136
15 6	427	3 26	40	132	135
16 7	505	3 86	39	141	136
16 3	526	3 87	38	146	134
15 8	531	3 88	38	152	132

the diuresis was constant again usually within 69—80 min Urine was then collected in 4—7 15 min periods and blood samples were drawn in the middle of each period as before

After completion of the water diuresis periods the volunteers were given an i v infusion of 3 % mannitol at a rate equal to the actual diuresis i e the infusion rate followed closely the diuresis during the preceeding urine collection period Urine was now collected in 10 min periods and as before blood samples were drawn in the middle of each period The study was stopped when the diuresis was again constant or when the subject started to feel discomfort Urine and serum samples were analyzed with regard to concentration of inulin PAH Na and K

He

an

Results

Table I demonstrates a protocol from a typical experiment In this case periods 6 through 8 are considered to be typical water diuresis recordings as judged by constant urine flow and constant low urine osmolalities At this time the diuresis had increased approximately 12 times The subjects generally tolerated the study well At no time there was any sign of overhydration

Hydropenia versus water diuresis

Table II presents a summary of the results from all subjects studied during hydropenia and water diuresis Water diuresis periods are selected as outlined above The mean values and standard deviations from all studies are given

TABLE II Summarized data from the transition from hydropenia to water diuresis

	GFR* ml/min		C_{PAH}* ml/min		$U_{Na}V$ µeq/min	
	H	W	H	W	H	W
A J	95	131	660	744	249	360
	100	111	738	668	262	312
		121		606		327
F A	83	103	526	461	134	111
	90	83	494	448	161	91
		98		451		101
L W	99	112	456	531	165	272
	88	110	476	567	171	241
		107		564		244
M H	92	90	610	610	91	10?
	114	84	621	549	84	82
	98		670		98	
B W	113	109	518	572	117	192
	113	100	520	514	133	154
	118		480		116	
A S	68	109	442	473	84	142
	78	119	482	533	95	149
		109		497		155
		103		529		153
S V	106	123	584	441	130	23
	99	129	571	468	133	221
	99	111	487	534	151	215
		119		484		271
n	17	21	17	21	17	21
M	97	109	549	535	140	184
S D	13 4	12 7	85	77	52	91
P	0 01	12 7	0 5		0 01	

* GFR and C_{PAH} calculated per 1 73 m² body surface

The glomerular filtration rate was increased in 5 of 7 subjects during water diuresis In contrast the clearance of PAH is little affected by the transition from hydropenia to water diuresis In only one subject L W was there a definite increase in the clearance of PAH during water diuresis In five of the seven subjects there was a definite increase in the urinary Na excretion The average fractional Na excretion increased from 1 01 % to 1 28 % during the transition from hydropenia to water diuresis In contrast there was a highly significant decrease in the urinary K excretion from 81 to 55 mEq/min when water diuresis instituted

The total osmolar clearance increased in all subjects studied during the transition from hydropenia to water diuresis from a mean of 2 13 ml/min to 3 57 ml/min

C_{Na}/C_{In} %		$U_K V$ µeq/min		C_{osm} ml/minC	
H	W	H	W	H	W
1 84	1 96	94	95	3 21	4 72
1 83	2 00	108	69	3 52	3 54
	1 93		77		3 39
1 10	0 77	53	66	1 85	3 33
1 23	0 83	75	52	2 34	3 01
	0 75		53		2 76
1 15	1 73	75	56	2 12	4 49
1 33	1 58	69	58	2 16	4 64
	1 63		59		4 42
0 68	0 82	107	47	1 71	2 59
0 51	0 71	110	38	1 74	2 30
0 70		114		2 04	
0 59	1 28	71	70	1 96	3 17
0 81	1 11	100	56	2.31	3 06
0 69		102		2 33	
0 88	0 96	38	17	1 27	3 18
0 88	0 94	37	16	1 40	3 49
	1 04		17		3 22
	1 07		17		4 28
0 88	1 39	80	81	2 01	3 95
0 96	1 25	71	74	1 99	3 68
1 08	1 31	76	73	2 20	3 60
	1 92		70		4 06
17	21	17	21	17	21
1 01	1 28	81	55	2 13	3 57
0 39	0 33	24	23	0 60	0 68
0 05 p	0 02	0 01 p		0 01 p	

The superimposition of osmotic load during water diuresis

This study was carried out to evaluate the effect of mannitol on free water formation i e distal tubular Na reabsorption In order to keep the hydration level as constant as possible and to reduce the number of exogenous variables mannitol was given in a dose approximately corresponding to the urine flow and without Na supplements The mannitol infusion during water diuresis did not appear to affect the renal hemodynamics considerably (Table III) The relationship between osmolar clearance and free water formation from 7 studies is illustrated in Fig 1 The osmolar clearance increased successively during the mannitol infusion The free water clearance at first increased as a function of the increasing osmolar clearance At C_{osm} of around 8 ml/

TABLE III Renal hemodynamics during water diuresis with superimposed mannitol load Values recorded 30—40 min following start of the mannitol infusion All values related to 1 73 m² body surface

	Water diuresis with superimposed mannitol load	Water diuresis only	p
	m ± S D	m ± S D	
GFR	106 ± 9 5	109 ± 12 7	>0 5
C_{PAH}	553 ± 53	535 ± 77	>0 5

100 ml GFR/min however the C_{H_2O} appeared to deflect and almost level off The urinary Na^+ excretion rose slowly as the osmolar clearance started to increase but increased more sharply when the osmolar clearance exceeded 8 ml/100 ml GFR/min *i e* at the deflection point of the free water clearance Since no extra Na^+ was supplemented with the mannitol, the subjects were slowly Na^+ depleted during the course of the mannitol infusion Towards the end of the study the Na depletion was even manifested by a slight fall in the serum Na^+ concentration

Discussion

The results have yielded significant differences between hydropenia and water diuresis with regard both to hemodynamics and tubular function The hemodynamic changes will be discussed first There is a slight but highly significant increase in the glomerular filtration rate during the transition from hydropenia to water diuresis This observation has been reported previously in man as well as in other species (Wesson *et al* 1950 Gilmore and Weisfeldt 1965) and has usually been attributed to extracellular volume expansion Definite proof of this theory is lacking It can not be excluded that other factors might at least in part contribute to the observed hemodynamic change It has been suggested that the distal tubular Na reabsorption plays an important role in the regulation of the renal vascular resistance in such a way that increased Na reabsorption will by some means decrease the renal vascular resistance (Aperia 1969 Although in the latter part of the discussion evidence will be presented for an enhanced distal tubular Na reabsorption relative to water reabsorption during water diuresis the results from the present study do not allow any further speculations on the mechanism of increased glomerular filtration rate during water diuresis *It is noteworthy that the PAH clearance remains unchanged* during the transition from hydropenia to water diuresis This might mean an actual redistribution of the renal blood flow However a reduced secretion capacity for PAH during water diuresis can not be ruled out on the basis of the present results

The significant differences in fractional Na excretion and osmolar clearance between hydropenia and water d[illegible] suggest certain discrepancies in the tubular handling of Na between the tw[illegible]retic states It is generally agreed that both in

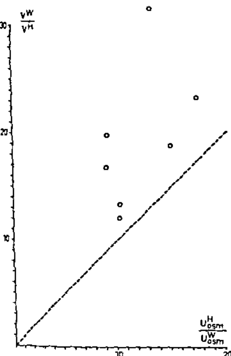


Fig 1

Fig 1 The relationship between osmolality clearai
C_{H_2O} and fractional Na excretion (C_{Na}/C_{In}) O
during the course of mannitol infusion

Fig 2 The relationship between expected (abscis
the transition from hydropenia to water diuresis
V^W urine flow ml/min during water diuresis
V^H ,, hydropenia
U^H_{osm} urine osmolality during hydropenia
U^W_{osm} , water diuresis

hydropenia and water diuresis the filtrate
passive reabsorption of water in the proxi
is delivered to the loop of Henle Here th
countercurrent system In the distal tubul
main difference between the handling
diuresis would then be found in the col
duct cells will be rendered water perm
osmotic equilibration between the collect
interstitium In water diuresis the collect
the final urine will approximately equal
Na excretion and osmolar clearance car
ADH on the water permeability of the
changes in the renal tubular handling of
Fig 2 If an identical filtrate would reac

water diuresis, a simple concentrating procedure during hydropenia would yield the following relationship

$$\frac{U^{H}_{osm}}{U^{W}_{osm}} = \frac{V^{W}}{V^{H}}$$

U^{H}_{osm}, urine osmolality during hydropenia
U^{W}_{osm}, » » » water diuresis
V^{H} , » volume » hydropenia
V^{W} , » » » water diuresis

In none of the subjects was this relationship achieved The urine volume quotient always exceeded the osmolality quotient Thus the discrepancy in urine flow between water diuresis and hydropenia is larger than what can be explained by a simple concentrating procedure only There are two immediate explanations for this observation

1 during water diuresis a reduced reabsorption in the proximal tubule of Na^{+} and water in isotonic proportions There are evidence that this occurs in the dog and in the rat kidney during water diuresis (Martino and Earley 1967 Hayslett *et al* 1967)

2 changes in solute reabsorption in the collecting ducts, *i e* an increased reabsorption of Na and urea during hydropenia This would be followed by passive water reabsorption There is in vitro evidence for an enhancing action of ADH also on the Na^{+} and urea permeability (Leaf 1967)

Both alternatives would yield a net increase in the osmolar clearance during water diuresis The results (table II) have already demonstrated a significant increase in osmolar clearance The relative importance of the two factors can be evaluated on the basis of the present results The total effect of the two aforementioned factors can however be further examined Both factors will provide an increase of osmolar clearance during water diuresis as compared to hydropenia If those were the sole determinants of the disproportionately high urine flow during water diuresis the disproportion would equal the differences in osmolar clearance between water diuresis and hydropenia and

$$\frac{U^{H}_{osm}}{U^{W}_{osm}} = \frac{V^{W} - (C^{W}_{osm} - C^{H}_{osm})}{V^{H}}$$

C^{W}_{osm} = osmolar clearance in water diuresis
C^{H}_{osm} = » » » hydropenia

This relationship has been examined in Fig 3 The figure demonstrates clearly that although the disproportion is reduced it is still found in all the patients studied Thus there must be an additional discrepancy between the tubular handling of Na and water in the two diuretic states

$$\text{If} \quad \frac{U^{H}_{osm}}{U^{W}_{osm}} \quad \frac{V^{W} - (C^{W}_{osm} - C^{H}_{osm})}{V^{H}}$$

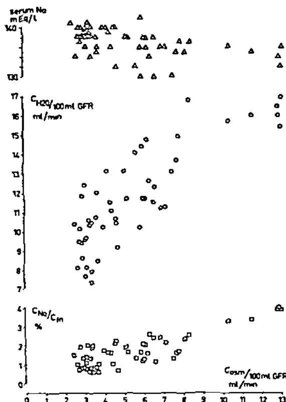


Fig 3 The relationship between expected (abscissa and actual (ordinate) dilution of urine during the transition from hydropenia to water diuresis when differences in osmotic clearances have been corrected for
C^{W}_{osm} osmolality clearance during hydropenia
C^{W}_{osm} osmolality clearance during water diuresis
Other symbols, see fig 2

was expected to be found, the assumption would have to be made that the fluid reaching the collecting tubules would be the same in hydropenia and water diuresis It might thus be possible that in man during hydropenia a certain water reabsorption would take place in the distal tubule, while during water diuresis only the corresponding amount of solute would be reabsorbed The delivery of free water into the collecting ducts would then be larger than during hydropenia This is probably the only situation when

$$\frac{V^{W}-(C^{W}_{osm}-C^{H}_{osm})}{V^{H}} \quad \text{would always exceed} \quad \frac{U^{H}_{osm}}{U^{W}_{osm}}$$

The analysis of the changes in urine osmolality osmolar clearance and urine flow during the transition from hydropenia to water diuresis thus strongly indicate that in man in opposite to dog the distal tubular cells are more water permeable during hydropenia than during water diuresis

Two possible explanations can be offered for the decreased urinary K excretion during the transition from hydropenia to water diuresis 1 An increased aldosterone secretion during hydropenia possibly as a result of contraction of the extracellular fluid volume 2) Increased Na concentration in the distal tubule during hydropenia facilitating the Na —K exchange

The recordings of the superimposition of osmotic load during water diuresis provide further information on distal tubular function in man It is generally agreed that mannitol by increasing the sodium gradient blocks the Na^+ reabsorption in the proximal tubule Thereby the Na^+ availability in the distal part of the nephron should be increased At the beginning of the mannitol diuresis there was a sharp *increase in the free water formation This is interpreted to be due to increased distal* tubular Na^+ reabsorption secondary to increased Na^+ availability in the distal tubular fluid When the maximal capacity for distal tubular Na^+ reabsorption under the present conditions is reached the increased Na^+ delivery from the proximal tubules can no longer be handled distally and, as a consequence of this Na^+ excretion starts to increase Good evidence for this interpretation are the simultaneous deflection points of free water formation and urinary Na^+ excretion as demonstrated in figure I

The present study has thus demonstrated a certain reserve capacity of the distal tubular Na^+ reabsorption It is however doubtful that the observed maximal distal tubular Na^+ reabsorption represents a true Tm It is more likely that any further increase of the distal tubular Na reabsorption is restricted by the transtubular Na *gradient and that the reabsorptive capacity could be further increased by a reduc*tion of the gradient Possibly this could be accomplished by supplementation of Na during the mannitol infusion

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Acta physiol. scand. 1969. 77. 439—441

From the Cardiovascular Research Institute and Department of Physiology, University of California, San Francisco, Medical Center, San Francisco, California, and White Mountain Research Station, University of California, Bishop, California, USA

Respiratory Sensitivity to Acute Hypoxia in Goat Kids Born at High Altitude

By

ALLAN H MINES and SOREN C SORENSEN

Received 19 February 1969

Abstract

MINES, A H and S C SORENSEN *Respiratory sensitivity to acute hypoxia in goat kids born at high altitude* Acta physiol scand 1969 77 439—441

Natives[1] of the Andean and Himalayan mountains have a lower respiratory sensitivity to acute hypoxia than do people born at sea level (Chiodi 1963, Severinghaus *et al* 1966, Milledge and Lahiri 1967 Sorensen and Severinghaus 1968a) We demonstrated that natives of the Andean mountains retain this lower sensivity even after many years of residence at sea level (Sorensen and Severinghaus 1968b), which indicated that the condition is irreversible However, we were not able to demonstrate any decrease in hypoxic sensitivity in sea level natives who lived for many years at high altitude during adult life (Sorensen and Severinghaus 1968a) The results suggested that irreversible loss of sensitivity to acute hypoxia might occur only through exposure to hypoxia during childhood In support of this conclusion we found that patients who were born with a congenital cyanotic heart disease (tetralogy of Steno Fallot) retained a low respiratory sensitivity to acute hypoxia 1—7 years after final correction of the cardiac malformation (Sorensen and Severinghaus 1968*c*) To obtain an animal model, we chose the goat because its respiration can be studied without anesthesia and we had already obtained appropriate control data in normal adult goats

[1] The use of the term "native" refers to the place of birth only

Methods

Two pregnant goats (1 Angora, 1 Nubian), 1—3 weeks before term, were brought from sea level to the Crooked Creek station of the University of California White Mountain laboratories at an altitude of 3100 m Their kids were kept at 3100 m for 6 weeks before being transferred to the Barcroft laboratory at an altitude of 3800 m where they were kept for 2 additional months, and then brought to sea level Their respiratory response to acute hypoxia was studied after being at sea level for 6—8 weeks At this time the Nubien kid weighed 15 kg, the Angora kid weighed 9 kg

The respiratory response to acute hypoxia was measured by determining the steady-state ventilatory response to CO_2 at two levels of P_{AO_2} (200 and 40 mm Hg) The kids were studied while standing in a stanchion, awake and lightly restrained Trial runs were conducted to accustom them to the experimental situation before final determinations were made The kids breathed [illegible]

manually diverted through a 37° °o Po_2 electrode (Radiometer, Copenhagen) by the operator watching the Pco_2 record $\dot{V}_E$ was measured by directing the expired volume into a Tissot spiro- [illegible] mber [illegible] The [illegible] els of [illegible] s were obtained

Results

Fig 1 illustrates the ventilatory response to CO_2 at end-tidal oxygen tensions of 200 and 40 mm Hg The changes in slope (S) of the CO_2 response expressed as the ratio S_{40}/S_{200} were 0 9 and 1 5 Pco_2 intercept at the zero ventilation fell 7 5 mm Hg and 2 2 mm Hg due to hypoxia

Discussion

In the 2 kids we studied, the respiratory sensitivity to acute hypoxia, as judged by the effect of hypoxia on the slope and the intercept of the CO_2 response curve was within the normal range we have found in awake, adult goats (Sorensen and Mines

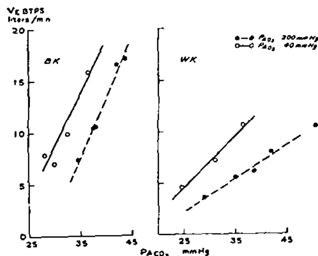


Fig 1 Ventilatory response to CO_2 at high and low alveolar oxygen tensions in 2 goat kids born at high altitude

1968d) Despite our small sample, it seems reasonable to conclude that we failed to produce in the goat respiratory insensitivity to acute hypoxia comparable to that in high altitude natives

Several explanations can be offered for this negative finding 1) Development of a low sensitivity to acute hypoxia under these conditions may cocur only in certain species 2) The duration of chronic hypoxia may have been too short for the development of a low ventilatory sensitivity to acute hypoxia 3) Fetal hypoxia may be involved in the development of a low respiratory sensitivity to acute hypoxia The mothers in the present study were brought to high altitude shortly before term The first explanation must be favored because a normal respiratory sensitivity to acute hypoxia has also been found in llamas and cats born at high altitude (Brooks and Tenney 1968, Hornbein and Sorensen unpublished)

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Acta physiol. scand. 1969. 77. 442—448

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The Unresponsiveness of Lipid Metabolism in Canine Mesenteric Adipose Tissue to Biogenic Amines and to Sympathetic Nerve Stimulation

By

KATHRYN BALLARD and SUNE ROSELL

Received 20 February 1969

Abstract

BALLARD, K and S ROSELL *The unresponsiveness of lipid metabolism in canine mesenteric adipose tissue to biogenic amines and to sympathetic nerve stimulation* Acta physiol scand 1969 77 442—448

The net release or uptake of glycerol and free fatty acids by adipose tissue in perfused mesentery of dogs was determined a) during stimulation of the superior mesenteric nerves and b) following intra arterial injection of noradrenaline, 5 hydroxytryptamine, histamine and compound 48/80 *Nerve stimulation had no observable effect on lipolysis, a finding which is in* contrast to previously reported results from subcutaneous adipose tissue Moreover in order to evoke lipolysis, noradrenaline and histamine had to be administered in amounts over 10 times higher than required for subcutaneous adipose tissue Compound 48/80 a mast cell-depleting agent, and 5 hydroxytryptamine did not increase lipolysis It is concluded that mesenteric adipose tissue may not be influenced by the same agents for lipolysis as subcutaneous adipose tissue from the same species

The perfused adipose tissue of the mesentery of dogs has been used to study the effect of sympathetic nerve stimulation and intra-arterially injected noradrenaline 5-hydroxytryptamine histamine and compound 48/80 on lipid metabolism The responses observed in this preparation were found to differ in several important aspects from those in subcutaneous adipose tissue from the same species (Rosell 1966) Factors previously shown to modify lipolysis in subcutaneous adipose tissue had slight or no observable effect on lipid metabolism in mesenteric fat

Methods

Mongrel dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg i v) Supplementary doses were administered throughout the experiment The data were obtained from 20 animals fasted for about 24 hrs Surgical preparation of the mesenteric adipose tissue was as follows The abdominal wall was opened along the *linea alba* extending from just below the sternum to about 5 cm below the umbilical scar A loop of small intestine (about 10—12 cm in length) was selected and the lymphatic tissue surrounding *the superior mesenteric* artery vein and peripheral nerves dissected away The nerve bundles were separated from the vessels tied and cut central to the ligature Branches from artery and vein other than

those supplying the selected loop were ligated The segment was isolated from the remainder of the intestine by successive cuts between ligatures Final separation of the mesentery of the isolated fan shaped section was accomplished by placing 2 ligatures around the vessels as they leave the mesentery to encircle the intestine, then cutting between the ties Extreme care was exercised to keep the tissue moist, warm and free of injury from manipulation Surgical preparation was completed and the recording of data was begun at approximately 1—2 PM

The tissue was perfused via the artery with defibrinated arterial blood taken from the same animal, a perfusion apparatus provided constant flow (Renkin and Rosell 1962) Perfusion pressure was recorded by a Statham transducer attached to the arterial inflow cannula Venous outflow was directed through a drop counter and then collected continuously, but in timed intervals, in ice-cold centrifuge tubes An arterial sample was obtained from the reservoir at the beginning and termination of each experiment, these samples provided a hematocrit, and an estimate of arterial plasma levels of free fatty acids (FFA) and glycerol in the perfusing blood Plasma from the collected venous outflow was also analysed for glycerol (Laurell and Tibbling 1966) and FFA (Trout, Estes and Friedberg 1960) The net release or uptake of [illegible] expressed [illegible] a Grass [illegible] approxi [illegible] se tissue [illegible] the rat [illegible]

Agents were injected via a side arm in the arterial inflow cannula Various doses were given but all solutions were administered in a total volume of 0 5 ml, or less, of physiological saline [illegible] tissue [illegible] 12/ [illegible] ction [illegible] oper- [illegible] give [illegible] nted through the drop counter and then returned to the femoral vein

Results

Sympathetic nerve stimulation In 12 trials from 8 dogs electrical stimulation of the distal end of the cut plexus of nerves failed to cause any change in the net release of glycerol or FFA from mesenteric adipose tissue Fig 1 illustrates one typical experiment The stimulation was applied at 10/sec for 5 min The net outflow or uptake of FFA was not significantly altered In most cases a slight vasoconstriction occurred

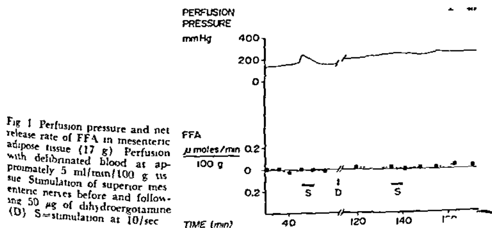


Fig 1 Perfusion pressure and net release rate of FFA in mesenteric adipose tissue (17 g) Perfusion with defibrinated blood at approximately 5 ml/min/100 g tissue Stimulation of superior mesenteric nerves before and following 50 µg of dihydroergotamine (D) S=stimulation at 10/sec

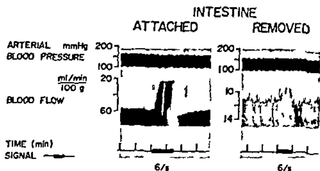


Fig 2 Effects of sympathetic nerve stimulation on blood flow in an autoperfused segment before and after the mesentery (14 g) was freed from the intestine Stimulation applied for one minute at 6/sec, 12 V Upper tracing shows blood pressure recorded from the femoral artery

as indicated by a change in perfusion pressure It is known that treatment of subcutaneous adipose tissue with α receptor blocking agents potentiates the release of FFA following sympathetic nerve stimulation (Fredholm and Rosell 1968) Therefore dihydroergotamine (50 μg) was injected i a (Fig 1) However, even following α receptor blockade there was no significant change in the release of FFA

The failure to observe any change in the release rate of FFA in the mesenteric adipose tissue during nerve stimulation might have been due to technical failure to activate the sympathetic nerves to the isolated section To examine this possibility the vascular responses to nerve stimulation were studied a) with the intestine attached and b) after the mesentery was freed Flow was first recorded during autoperfusion with the intestine still attached (Fig 2 left) Pronounced vasoconstriction occurred during stimulation of the nerve plexus The flow response to the same type of stimulation after removal of the intestine was greatly reduced (Fig 2, right) Fig 3 shows a plot of the flow rates during a control period (clear bar) and during nerve stimulation Control blood flow in the segment was 54 ml/min/100 g with the intestine attached Stimulation of the nerve bundle at 6/sec decreased the rate of flow to 21 ml/min/100 g a reduction of about 60 per cent On the other hand after the intestine was removed the flow during the control period and time of stimulation

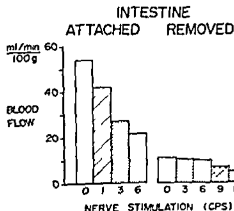


Fig 3 Effects of sympathetic nerve stimulation on blood flow in perfused intestinal segments before and after the mesentary is freed Stimulation was applied for 3—5 min for each trial The white bars show the resting blood flow The bars indicate the mean values from three experiments

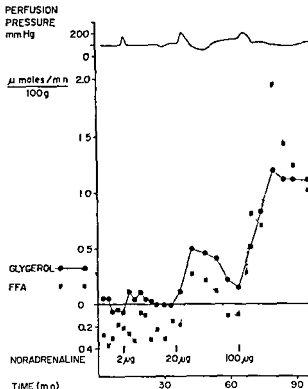


Fig 4 Perfusion pressure and net release rates of glycerol and FFA in mestenteric adipose tissue (12 g) Perfusion with defibrinated blood at 8 ml/min/100 g tissue Repeated doses of noradrenaline were given intra arterially

was 11 and 10 ml/min/100 g respectively In this case the reduction was insignificant It appears therefore that the response of the intestinal vascular bed to sympathetic nerve stimulation was relatively much stronger than that of the associated mesenteric tissue Similarly the sympathetic regulation of lipolysis in the mesentery may also be of minor importance

Noradrenaline administration increased the net release of FFA and glycerol from mesenteric adipose tissue (5 out of 13 trials in 5 dogs) However, only high doses were consistently effective Fig 4 shows the response to doses ranging from 2 μg to 100 μg No significant increases in lipolysis were observed with amounts below 20 μg At the highest dose 100 μg nearly 2 μmoles/min/100 g FFA and 1 1 μmoles/min/100 g of glycerol were released On the other hand vasoconstriction occurred when 2 μg of noradrenaline were given as shown by the perfusion pressure plotted in the upper tracing on the figure In other experiments vasoconstriction was observed with doses ranging from 0 25 μg to 5 0 μg without any concomitant net release of glycerol and FFA

Histamine has been shown to enhance lipolysis in subcutaneous adipose tissue (Fredholm Meng and Rosell 1968) In 13 out of 16 injections from 8 preparations of the mesenteric fat the net release of glycerol and FFA was consistently incre

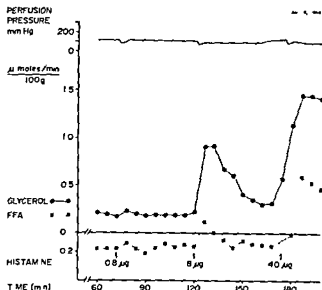


Fig 5 Perfusion pressure and net release rates of glycerol and FFA in mesenteric adipose tissue (32 g) Repeated doses of histamine given intra arterially Perfusion with defibrinated blood at 3 ml/min/100 g tissue

by amounts of histamine above 8 μg A representative experiment is shown in Fig 5 The output of glycerol was increased from a basal net release of 0 2 μmoles/min/100 g to 0 9 following injection of 8 μg histamine A comparable change in the levels of FFA likewise occurred A constant net uptake of 0 15 μmoles/min/100 g was reversed to a net release of 0 13 μmoles/min A higher dose of histamine (40 μg) resulted in a more pronounced effect Compared to subcutaneous adipose tissue much higher doses are required to elicit the same response in mesenteric tissue The threshold dose for subcutaneous tissue was 0 5 μg the mesenteric adipose tissue required 8 μg for a consistent minimal response

Compound 48/80 a histamine releasing agent (Paton 1951 Hogberg and Uvnäs 1957) had a minor effect in only three out of 19 trials (8 dogs) in mesenteric tissue Concentrations ranging from 10 to 500 μg were injected This agent increases the net release of glycerol and FFA from subcutaneous adipose tissue (Fredholm Meng and Rosell 1968)

5 hydroxytryptamine (5 HT) a biogenic amine shown to increase plasma FFA in dogs and man (Carlson Ekelund and Oro 1967) had no observable lipolytic effect on the mesentery with dose as high as 100 μg (9 trials in 4 dogs) This is also the case in canine subcutaneous adipose tissue

Discussion

The consistent failure of sympathetic nerve stimulation to induce FFA mobilization from mesenteric adipose tissue constitutes an important difference in comparison with the responses in subcutaneous adipose tissue It is also interesting to note that the influence of sympathetic stimulation on blood flow was much greater on the in

testinal portion of the unit than on the associated adipose tissue (Fig 2) The autonomic nerve activity thus appears to have a lesser effect on the circulation in the mesenteric portion at least at stimulation frequencies considered to be within the physiological range (Folkow 1952) Lipid metabolism may likewise not be modulated by nervous influences Possibly related to the failure of sympathetic nerve stimulation to increase lipolysis in mesenteric adipose tissue is the weak response to low or moderate doses of noradrenaline It was necessary to use very high doses of noradrenaline in order to increase lipolysis The threshold dose for enhanced lipolysis in subcutaneous adipose tissue is about 0 1 μg (unpublished results) whereas similar changes in mesenteric adipose tissue were consistent only when amounts above 20 μg were injected Thus the influence of either added or released noradrenaline on the mesentery is minimal at levels shown to be effective in adipose tissue from a different site in the same species

It has previously been suggested that adipose tissue from different localities shows quantitative differences of response to noradrenaline (as well as to adrenaline) Aronovsky *et al* 1963, reported that adipose tissue *in vitro* obtained from the orbital socket subcutaneous areas omentum and paw of the cat each responded differently to catecholamines Carlson and Hallberg 1968 observed that the amount of glycerol released *in vitro* by 1 μg/ml of noradrenaline was significantly greater in omental than in subcutaneous adipose tissue of human origin

Compound 48/80 failed to increase lipolysis in mesenteric fat in contrast to the effect in subcutaneous adipose tissue This was somewhat unexpected since in both subcutaneous and mesenteric canine adipose tissue histamine increased the release of FFA One would expect similar responses to a mast cell degranulating agent The threshold dose of histamine to stimulate lipolysis was rather high It would therefore be reasonable to assume that the dose of compound 48/80 must be comparably elevated However concentrations as high as 500 μg of compound 48/80 had no effect on lipolysis in mesenteric adipose tissue

The mesenteric tissue in the dog might not contain a sufficient number of mast cells to release the high concentration of histamine necessary to stimulate On the other hand it has been reported that the mesentery of rats contains mast cells (Riley and West 1953 Gustafsson and Cronberg 1961 Fernex 1968 which are disrupted in the presence of compound 48/80 Hogberg and Uvnas 1957 Gustafsson and Cronberg 1957) However at this writing we are unaware of data giving a quantitative estimate of number of mast cells present in the canine mesentery

The present experiments clearly indicate that there are quantitative as well as qualitative differences in the control of lipolysis in adipose tissue from different locations Therefore we suggest that mesenteric adipose tissue is not influenced by the same regulatory mechanisms for lipolysis as subcutaneous adipose tissue from the same species

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Acta physiol. scand. 1969 77. 449—464

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Mast Cells and 5-HT. Intracellular Release of 5-Hydroxytryptamine (5-HT) from Storage Granules during Anaphylaxis or Treatment with Compound 48/80

By

S A CARLSSON and M RITZEN

Received 21 February 1969

Abstract

CARLSSON, S A and M RITZEN *Mast cells and 5 HT Intracellular release of 5-hydroxytryptamine (5 HT) from storages granules during anaphylaxis or treatment with compound 48/80* Acta physiol scand 1969 77 449—464

The release of 5 hydroxytryptamine (5 HT) and granules from peritoneal mast cells during *in vitro* and *in vivo* anaphylaxis and after treatment with compound 48/80 *in vitro* was investigated by quantitative cytochemical methods Sensitization was achieved by s c injections of horse serum with *H pertussis* and anaphylaxis was observed 3—30 weeks later The 5 HT content of mast cells was assayed by microfluorimetry the cellular content of granules by microinterferometric measurements of cellular dry mass Incubation of peritoneal mast cells with antigen *in vitro* resulted in release of 7—32 % of 5 HT compared to 20—50 % release following i v injection of specific antigen to the same rat Incubation of non sensitized mast cells with compound 48/80 (1 6 μg/ml) caused a release of 45 % of the 5 HT Microfluorimetric and microinterferometric measurements of the same individual mast cells revealed a proportionally greater release of 5 HT than lowering of dry mass values Since only a small part of the cytoplasmic dry mass is non granular material the results indicate that under the present experimental conditions about 40 % of the total 5 HT release was not accompanied by a simultaneous extrusion of granules It could also be demonstrated by fluorescence microscopy that a varying proportion of the granules had released their 5 HT while still intracellularly located This demonstration of intracelular release of 5 HT from storage granules may be relevant also for other types of secretory cells

Mast cells have long been of primary interest in attempts to elucidate the basic mechanisms underlying the anaphylactic shock Thus it is known from combined pharmacological and histological studies that histamine and 5 hydroxytryptamine (5 HT) release from tissues during anaphylaxis is accompanied by mast cell damage However the conventional histological techniques for staining mast cells provide information on cell structure and possibly heparin content but it has not been possible to determine the amounts of biogenic amines present in the cells Recently the Falck Hillarp method for the histochemical demonstration of monoamines has been investigated and standardized so as to permit quantitative measurements of the 5

TABLE I The results of 8 expts on the antigen induced release of 5-HT from rat peritoneal mast cells (per cent release compared to control mast cells from the same rat) Peritoneal cells were incubated with antigen for 30 min, and the rats were killed 15 min after injection In experiment no VIII the rats were sensitized only 14 days before anaphylaxis, in all other experiments more than 21 days (See text!) Figures within parenthesis show release from control unsensitized littermates An —before figure denotes increased fluorescence.

Exp	Rat	% release In vitro	% release In vivo	Exp	Rat	% release In vitro	% release In vivo
I	a	27***	——	V	a	7	——
	b	17**	——		b	17**	36***
	(c	—9	——)		(c	—16	—10)
II	a	23***	——	VI	a	13	20**
	(b	8	——)		b	18*	46***
III	a	32***	42***	VII	a	——	50***
	b	21***	31***	VIII	a	2	——
	(c	4	1)		b	—13	——
IV	a	28***	——		c	—18**	——
	b	9	——		(d	—15	——)
	(c	—6	——)				

$* = p < 0.1$, $** = p < 0.05$, $*** = p < 0.01$ (Student's t test)

same antigen, the loss of fluorescence found ranged between 7—32 and 20—50 % respectively (Table I) The mast cells of control littermates never showed any reaction to antigen injections However, there was a recurring tendency of the mean mast cell fluorescence of these rats to increase by a few per cent following antigen treatment, possibly due to an unspecific effect of the added serum Even in the sensitized rats a few mast cells could always be found that contained up to the maximal amount of 5-HT found in the control cells These cells also retained their normal shape and showed no signs of damage

No evidence was found for a separation of the mast cell population into reacting and nonreacting cells Judging from the frequency histograms (Fig 2a) and the morphology of the cells most mast cells were affected by the antigen

Dry mass The total dry mass of a mast cell is made up of three different parts, the granules the non-granular cytoplasm and the nucleus Ottoson *et al* (1957) measured the relative size of the nucleus of mast cells and found the radius of the nucleus to be 35 % of that of the whole cell Thus, the nucleus constitutes about 4 % of the volume of the cell In the interference microscope the same authors found the optical path through the nucleus to be only 80 % of that through the cytoplasm adjacent to the nucleus With the geometrical figures given this correspond to a concentration of solids in the nucleus which is 29 % of that of the cytoplasm Thus, the dry mass of the nucleus is only around 1 3 % of the total dry mass

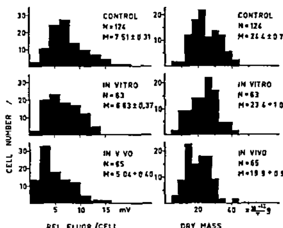


Fig 2 H stograms showing the fre quency distribution of fluorescence in tensity and dry mass of peritoneal mast cells after *in vitro* and *in vivo* anaphylax s in the same rat Means ± S E M and number of cells measured (N) Only for the *in vivo* anaphylaxis is there a s gnificant ($p < 0 01$) de crease in dry mass No subgroups re acting differently within the popula t on can be distinguished neither with respect to fluorescence nor to dry mass

The cytoplasm of the normal mast cell consists of densely packed granules and non granular cytoplasm in between (see electron micrographs by Bloom and Haeger mark 196o) The relative dry mass of these compartments will be determined by their relative volume and density In maximal hexagonal packing of equally sized spheres the latter occupy 74 % of the total volume independent of size When the spheres are of varying size, like mast cell granules they take a considerably larger part of the volume The mean total concentration of solids in normal peritoneal mast cells was shown by Diamant and Lowry (1966) to be 47 6 % The concentra tion of solids in the cytoplasm has been measured in the case of human epithelial cells which contain few dense cytoplasmic organelles to be 14—18 % (Barer 19o3) Assuming that 16 % is an approximately correct figure for mast cell non granular cytoplasm the concentration of solids in the granules equals 59 % In the case of maximal hexagonal packing of equally sized granules the ratio granular dry mass to total cytoplasmic dry mass will be 0 94 Subtracting the nuclear part of the dry mass (1 % see above) the granules constitute 93 % of the total dry mass of the cell This figure is evidently an approximation it will be higher if the varying size of the granules is considered lower if many cells have relatively few granules However due to the large difference in density of the granules and the non granular cytoplasm the granules will still constitute the major part of the dry mass

The dry mass of the control mast cells of this experiment (rats weighing 120 g) averaged $24\ 4 \times \frac{10^{-12}}{\varphi}$ cm³ Assuming $\varphi = 0\ 077$ cm³/g (Carlsson 196o see also Ritzen 1967 c) this gives a mean weight of $3\ 17 \times 10^{-1}$ g After *in vivo* anaphylaxis there was a significant decrease in the mean dry mass of the mast cells (Fig 2b The magnitude of this decrease was however smaller than that of 5 HT when the two parameters were measured on the same cells (Table II) When the 5 HT content and the dry mass were compared the control cells showed a very good correlation between the two parameters (Fig 3a) Judging from the changed morph

TABLE II. The release of 5-HT and decrease in dry mass of rat peritoneal mast cells following 15—30 min treatment with antigen (actively sensitized mast cells) or compound 48/80. Control, *in vitro* and *in vivo* anaphylactic cells were obtained from the same rat. (See text.) "Granular" and "nongranular" 5-HT release denote the percentage of the total decrease in fluorescence that respectively could and could not be ascribed the decrease in dry mass of the same cells. n = number of cells measured. flour = mean fluorescence per mast cell ± SEM (arbitraty units), dry mass = mean dry mass per mast cell ± SEM, $\left(\times \frac{10^{-13}}{g}\right.$ gram (see text)$\left.\right)$

	Antigen			48/80	
	In vitro	In vivo	Control	48/80	Control
n	63	65	124	96	98
Fluor	6.63 ± 0,37	5,01 ± 0.40	7,51 ± 0.31	14.0 ± 0,8	25,7 ± 1.2
% of control	88.3*	67,1***		54,5***	
Dry Mass	23,4 ± 1.0	19,9 ± 0,9	24,4 ± 0.7	21,8 ± 1.0	31,8 ± 1,2
% of control	95,9	81.6***		71,3***	
granular 5-HT release, %		56		63	
nongranular 5-HT release, %		44		37	

* p < 0.1, ** = P < 0.05, *** = p < 0.01 (Student's t test)

mast cell population seemed to have reacted in a quite homogenous manner, with few exceptions both large and small cells were affected. When the material was divided into classes with respect to dry mass a significant difference in fluorescence intensity ($p<0.01$) between *in vivo* anaphylactic cells and control cells was found *for the small cells (after the release process) (Fig. 3c). This means that the concentration* of 5-HT (5-HT per unit dry mass) has decreased in mast cells of this size while in the other cells the 5-HT and dry mass had decreased at about the same

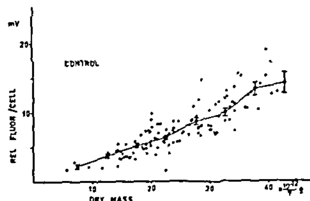


Fig. 3a. Fluorescence versus dry mass of individual control peritoneal rat mast cells. Each point represents one mast cell. Also class means ± S.E.M. are shown. The two parameters show a good correlation.

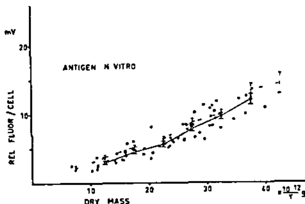


Fig 3b Rat peritoneal mast cells from the same sensitized rat as Fig 3a but after *in vitro* challenge with specific antigen The curve for the control cells is dashed When compared to the controls there is no significant change in the concentration of 5 HT in the dried cells (the ratio fluorescence/dry mass is almost the same)

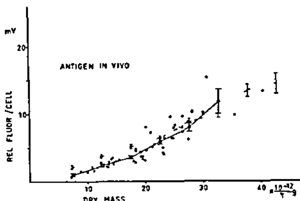


Fig 3c Fluorescence versus dry mass of peritoneal mast cells from the same rat as Fig 3a and 3b These cells were collected after i v injection of specific antigen Both fluorescence and dry mass are decreased compared to control cells (dashed line) but the concentration of 5 HT within the dried cells is significantly decreased only for the smallest cells

rate The small decrease in dry mass of mast cells after *in vitro* treatment with antigen was not statistically significant (Table II Fig 3b)

Morphology The smears of antigen treated cells only showed a few mast cells that seemed to be quite unaffected by the treatment they exhibited a brilliant yellow fluorescence In the control cells a few small non fluorescent vacuoles could occasionally be observed in cells that had been flattened out on glass while most cells had a shape of a fluorescent ball with indistinguishable substructures (Fig 4) In the *in vitro*, and even more in the *in vivo* antigen treated cells the number and sizes of such non fluorescent vacuoles increased considerably and often several granules were seen clumped together into a very large vacuole (Fig 5) After subsequent fluorescence microphotography and staining with toluidine blue the non fluorescent vacuoles were found to contain metachromatically stained granules However it was not possible in these spread specimens to exclude the possibility that these granules had already been extruded from the cell and actually were extracellular in position and merely adhered to the surface of the mother cell Such a fenomenon has been demonstrated during the action of compound 48/80 on mast cells (Thon and Uvnas 1967) Therefore cell pellets were freeze dried treated with paraformaldehyde em



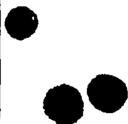


Fig. 4. Fluorescence and light micrographs of three normal mast cells, smeared on glass from a suspension of control peritoneal cells. Smears were dried, treated with formaldehyde gas, photographed and stained in toluidine blue. Fluorescence indicates presence of 5-HT (left micrograph), toluidine blue stains mainly heparin (right). Note that the cells are relatively thick, so that individual granules are hardly distinguished. 1080×.

bedded in Araldite®, and sectioned at about 1 µm. In these sections, the relative 5-HT content of each granule in the cell could be observed in the fluorescence microscope, and after subsequent staining in toluidine blue, the same granules could be identified as intra- or extracellular.

Several degrees of alteration of the mast cells could be seen in the antigen-treated specimens. In the fluorescence microscope, some cells showed a varying number of large, non-fluorescent or very weakly fluorescent vacuoles. These could always be identified in the stained sections as containing metachromatic granules. No empty vacuoles were seen. The "altered" granules were always larger and stained less intensely and more red than the blue to violet staining normal, 5-HT containing granules. In mast cells that had released most of their 5-HT large vacuoles were often seen in the fluorescence microscope (Fig. 5, 6). After staining these large vacuoles were found to contain a conglomerate of granules that had lost their 5-HT. The plastic sections revealed their true intracellular location. Some cells totally lacked



Fig. 5a. Mast cells from a peritoneal cell suspension from an actively sensitized rat. The cells were treated *in vitro* with specific antigen (horse serum). Fixation and staining as in Fig. 4. Note that one of the mast cells has a normal morphology and 5-HT content while the other contains only a few fluorescent granules.

Fig. 5b. Same specimen as Fig. 5a. The lower cell has developed a large nonfluorescent vacuole staining distinctly with toluidine blue (the lower nonfluorescent vacuole is the cell nucleus). The upper mast cell is devoid of 5-HT and has only a few granules left in the cytoplasm. 1320×. Formaldehyde and toluidine blue.

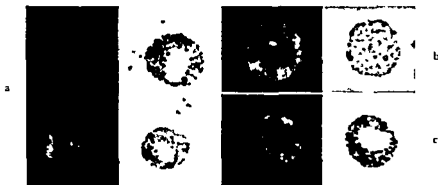


Fig 6a. A thin (0 5—1 μm) plastic section of rat peritoneal mast cells treated *in vitro* with specific antigen Due to the thinness of the section the fluorescence of most granules is weak Several totally nonfluorescent granules may be seen by comparing the fluorescence and the light micrographs

Fig 6b Same specimen as Fig 6a plastic section Most granules have a very faint fluorescence The granules are increased in size, and the whole cell seems to be uniformly emptied of 5-HT (The nonfluorescent vacuoles observed in Fig 5 and 6a here occupy the whole cytoplasm)

Fig 6c Control mast cells from the same rat as Fig 6a and 6b, after incubation with isotonic salt solution only The granules can be outlined by their fluorescence and they stain distinctly with toluidine blue Note the difference in size between the granules of the cells in Fig 6b and 6c 1320× Formaldehyde and toluidine blue

strongly fluorescent granules In the antigen treated cells, very few disintegrated mast cells were seen Also, relatively few granules were seen occurring free in the space between the cells No *fluorescent* granules could be seen extracellularly

After completed polymerization of the plastic, the normally non-fluorescent red blood cells showed a blueish fluorescence which increased somewhat upon ultra violet irradiation This fluorescence was easily distinguished from that of 5 HT

48/80

5 Hydroxytryptamine It has repeatedly been found that mast cells of old rats of this strain contain more 5-HT than those of young animals Therefore the fluorescence of these mast cells was stronger than that of the younger rats used for the ex periments above Following incubation with compound 48/80 at a concentration of 1 6 μg/ml many cells showed marked alterations and the mean fluorescence dropped to around 55 % of the control cells Also in this case most of the cells participated in the reaction (Table II Fig 7a) The results were similar to those obtained in earlier experiments with compound 48/80 (Ritzen 1967 a)

Dry mass Peritoneal mast cells of old rats are larger than those of young rats (Padawer 1963) Thus the mean dry mass of the mast cells of the 48/80 experi ments was higher than that of the antigen experiments where younger rats were used These large control cells showed a mean dry weight of $34\ 8 \times \frac{10^{-11}}{\varphi}$ cm³ (equals $4\ 4 \times 10^{-10}$ g if $\varphi = 0\ 077$ cm³/g, see above) Following treatment with compound 48/80, the mean dry mass decreased by about 30 % while the 5 HT decrease

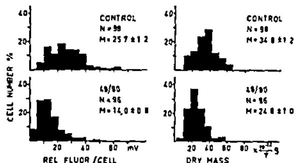


Fig 7 Histograms showing the frequency distribution of fluorescence (left) and dry mass (right) of peritoneal mast cells after treatment with compound 48/80 concentration 1 µg/ml Both parameters are significantly decreased (p<0 01)

amounted to 45 % (Table II, Fig 7) Thus also in this case, there was a discrepancy between the release of dry mass (granules) and 5-HT

Mast cells from different rats have different amounts of 5-HT In the experiment with compound 48/80, peritoneal cells from 4 rats were pooled, and therefore the individual cells show a considerable deviation from the mean (Fig 8 a, b)

No distinct subgroups within the population as regards reactivity to compound 48/80 were observed (Fig 7), although the decrease in concentration of 5-HT in the cells was most evident for cells of middle size (20—40 mass units) after treatment (p<0 01)

Morphology In the spreads of peritoneal cells, the same general picture as described above for antigen treated cells could be seen in the fluorescence microscope However damage to the cells was more pronounced, and some cell ghosts containing only a few granules were seen In cells that had retained their general shape the distribution of fluorescent granules within the cytoplasm was not uniform Very often cells were seen where most of the cytoplasm was occupied by one or more non

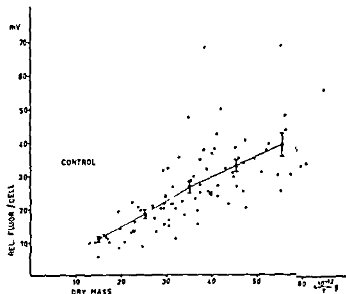


Fig 8 a Fluorescence versus dry mass of individual rat peritoneal mast cells from control sample The older age of this rat compared to that in Fig 3 is revealed as a larger size and stronger fluorescence of the mast cell Still there is a good correlation between the parameters Class means ± S E M are shown

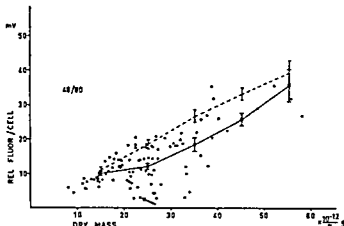


Fig 8b Same cell suspension as in Fig 8a, after treatment with compound 48/80 Both dry mass and fluorescence have decreased leaving only few cells that are still large and strongly fluorescent The concentration of 5 HT in the dried cell decreased significantly compared to the control cells (dashed line) in the middle size range ($p < 0.01$) The arrow points at the cell shown in Fig 9a

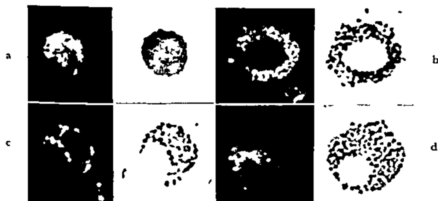


Fig 9a Rat peritoneal mast cell treated with compound 48/80 This cell is indicated by arrow in Fig 8b The large 'vacuoles' filled with nonfluorescent granules explain the low total concentration of 5 HT in the cell Formaldehyde treated peritoneal smear, stained with toluidine blue after fluorescence microscopy 1320 ×

Fig 9b A thin (0 5—1 μm) section from a 48/80 treated peritoneal cell sample after formaldehyde treatment and plastic embedding Most of the granules have retained their fluorescence, but in some areas of the cytoplasm the granules have lost its 5 HT enlarged and become pale (pink to red) staining in toluidine blue 1320 ×

Fig 9c Thin section from the value sample as Fig 9b This cell is in a later stage of the release process, showing large vacuoles containing nonfluorescent large pale staining granules 1320 × Formaldehyde and toluidine blue

Fig 9d Th
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fluorescent areas appearing as vacuoles in the fluorescence microscope However after staining, these vacuoles turned out to be filled by metachromatic granules (Fig 9a) The intracellular location was confirmed in studies of plastic sections The altered granules stained weaker and more red than the normal granules, appearing gray in the micrographs (Fig 9b c, d) Very often, a clear unstained halo could be

seen surrounding the non fluorescent altered granules In the plastic sections of the compound 48/80 treated cell suspensions large amounts of red non fluorescent granules were seen between the cells Also, some apparently bursted cells could be seen where some granules still stuck to the nucleus while the periphery of the cell was disintegrated These cells could not be located in the fluorescence microscope before staining

Assuming that the decrease in dry mass of the mast cells is due to a release of granules and that the 5 HT of these granules leave the cell concomitantly a rough calculation of the relative magnitude of granular and nongranular 5 HT release at the moment of fixation could be made (Table II) In the *in vivo* treatment of mast cells with antigen there was a release of 33 % of 5 HT but only 18 % of dry mass Thus the loss of granules would account for 56 % of the total 5 HT release the rest being lost without mass decrease Similar calculations for the experiments with compound 48/80 show a somewhat larger proportion of granular 5 HT release (63 %)

Discussion

It has been shown (Moran *et al* 1962, Provost Danon *et al* 1966) that histamine and 5 HT are released from rat and mouse mast cells almost simultaneously There is nothing to indicate that the two amines are stored in different cellular structures On the contrary there is evidence indicating that they compete for the same binding sites in the peritoneal rat mast cells (Cabut and Haegermark 1966) Thus at present it seems justified to discuss some aspects of histamine and 5 HT storage and release in the same context

Controversial reports are found in the literature concerning the release of biogenic amines from actively sensitized rat peritoneal mast cells upon treatment with antigen *in vitro* Thus some authors (Humphrey *et al* 1963 Keller 1966) do not find any significant histamine release under *in vitro* conditions while others (Uvnäs and Thon 1959 Perera and Mongar 1963 Mota 1964 Schwartz and Vardinon 1964) obtain a reproducable release up to 62 % It has been suggested that this is due to differences in the rat strains used (Binaghi 1968) or the modes of sensitization In the present investigation it was unexpectedly found that a consistent mast cell response to the antigen was only obtained when a relatively long time had elapsed from the time of sensitization (more than 3 weeks) In spite of a lethal anaphylactic shock following i v challenge with the antigen on day 8—20 after the first sensitizing injection no or very little mast cell damage or 5 HT release could be observed From 3 weeks and up to at least 3 months 5 HT release was consistantly noted This is surprising since after 3 weeks the titer of homocytotropic antibody in serum is declining (Mota 1963 Bloch 1968 Evidently cell bound antibodies remain for a much longer time One out of several experiments with negative result is presented Table I exp VIII where the *in vitro* anaphylaxis of mast cells was tested 14 days after sensitization No release of 5 HT can be noted On the contrary as in unsensitized control rats the fluorescence of the mast cells showed a slight *increase* In

one rat the increase was statistically significant as compared to control cells The reason for this paradoxal affect of the addition of serum cannot be explained at present

Even at an appropriate time after sensitization the response of the rats was not of the same magnitude varying even between littermates treated identically (Table I) No strict correlation between clinical symptoms and peritoneal mast cell damage could be seen Mast cells in mesenterial spreads were generally found to be more easily damaged than the free peritoneal mast cells This might very well be a result of the mechanical trauma to the cells that was necessary when preparing spreads of the mesenterial pieces The cells treated with antigen (or compound 48/80) were often flattened out on the glass giving the impression of impared elasticity of the cytoplasmic membrane

The present findings support the view that antigens and compound 48/80 act similarly on rat peritoneal mast cells Apart from the fact that the effects of 48/80 treatment were somewhat easier to reproduce no distinct differences were noted in the morphology of the mast cells or the intracellular distribution of 5 HT when the effects of the two liberators were compared This is in agreement with recent findings of Bloom and Chakravarty (personal communication) who have studied the anaphylactic reaction in rat peritoneal mast cells with the aid of the electron microscope and compared the results with the effects of 48/80 on mast cell fine structure (Bloom and Haegermark 1965)

No real attempt to quantitate granular release from mast cells during histamine or 5 HT release has been published probably due to difficulties in separating the cells from released granules with conventional biochemical methods Interference microscopy has been used for this purpose by Ottoson *et al* (1957) but with the methods available they could not observe any decrease in dry mass of the mast cells measured in spite of a marked histamine release following treatment with compound 48/80 The present investigation showed a significant decrease in dry mass of mast cells following both *in vivo* anaphylaxis and treatment with compound 48/80 (Table II) The small decrease in dry mass following *in vitro* incubation with antigen (4%) was not statistically significant The tendency of released granules to stick to the mast cells *extracellularly* which has been described in direct observations of the mast cells during the release processes (Thon and Uvnas 1967) could not be seen in the present specimens However it is possible that released granules were detached during the centrifugation and resuspension of the cells before preparing the specimens As can be seen in the plastic sections the mast cells were well defined and the dry mass measured did not include adhering extracellular granules

The methods used in the present investigation makes it possible to compare the decrease in dry mass of the cells with the release of 5 HT from the same cells In this way the part of the 5 HT release that may be accounted for by the release of granules (dry mass) may be calculated and thus the proportions of granular and [illegible] granular 5 HT release Such calculations (Table II) indicate that with [illegible] total 5 HT release the proportion of granular release increased up [illegible]

60 % (in case of 48/80 treatment) Thus, the "non-granular" 5-HT release seems to be of a considerable magnitude, more than was expected The results reflect the situation 15—30 min after addition of the releasing agent At the moment of 5 HT release the relative proportions of "granular" and "non-granular" release probably were different Some (or all) of the 5-HT release that is confined to the "granular release might have left the cell before the granule, and thus at this moment, the figure for "non-granular' release is rather an underestimate

As mentioned earlier, the non granular part of the cellular dry weight might introduce an error in cells that have released most of their granules, when dry mass is used as a measure of amount of granular material However, in the 48/80 experiment calculations from the figures given show that the large difference between the 5 HT and the dry mass decrease could only be explained in this way if the non granular part of the normal mast cells amounted to 36 % of the total dry weight This is highly improbable (See calculations under "results" above!)

The morphological features of 5-HT release following treatment with antigens or *compound 48/80 are in agreement with the results of the quantitative measurements* Since the cells were observed 15 and 30 min after the beginning of the treatment the various appearances of the cells reflect different degrees of 5 HT release rather than different stages of release In spite of the large number of non-fluorescent granules often seen intracellularly, no evidence of 5-HT deposition in the cytoplasm between the granules was ever found Thus, after being released from granules 5-HT rapidly leaves the cell

The present cytochemical and morphological findings fit very well into the electron microscopical picture seen after 48/80 treatment of rat peritoneal cells (Bloom and Haegermark 1965 Horsfield 1965) Bloom and Haegermark (1965), with the aid of the electron microscope described in detail various stages in mast cell granule 'alteration" as well as extrusion of granules during histamine release induced by compound 48/80 The altered granules showed a marked decrease in electron density after fixation in glutaraldehyde and osmiumtetroxide and it was suggested by these authors that the granule alteration was due to loss of histamine and possibly some other component(s) from the granules They also observed that altered granules were frequently gathered in larger well delimited, intracellular vacuoles It was also shown by these authors that in epon sections stained with toluidine blue and examined in the light microscope granule alteration was reflected in an increased metachromasia of such granules They suggested that the removal of e g basic histamine would increase the number of acidic polysaccharide groups available for dye-chromotrope interaction

It is well-known from earlier studies that a large part of the granules are released from the mast cell following contact with releasing agents (Stuart 1952, Mota 1953 Hogberg and Uvnas 1957 a o and it has been emphasized that degranulation is the principle (if not only) way of release of biogenic amines from mast cells (Thon and Uvnas 1967) This view is supported by the finding that when compound 48/80 exerts its effects on mast cells in media poor in cations (e g isotonic sucrose), 50 %

of the released histamine is still bound to granules *extra* cellularly (Thon and Uvnäs 1967) Thus, the displacement of histamine at the heparin—protein complex by cations (Åberg *et al* 1967) needs extracellular cations Whether the experimental conditions with cation poor media induce another type of 5 HT release from mast cells than the ionic milieu used in the present investigation is being further studied in our laboratory

In conclusion the present experiments show that mast cells exposed to antigen or compound 48/80 release a considerable part of their 5 HT without a concomitant release of granules Thus degranulation does not seem to be obligatory for release of the stored biogenic amine

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Studies on Central Regulation of Secretion of Antidiuretic Hormone (ADH) in the Goat

By

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Received 24 February 1969

Abstract

OLSSON, K. *Studies on central regulation of secretion of antidiuretic hormone (ADH) in the goat* Acta physiol scand 1969 77 465—474

A further analysis was made of the antidiuretic response to infusions of NH_4Cl *into the 3rd* brain ventricle of hydrated goats The response was obtained in the absence of "osmotic" stimulation, but was evidently due to a release of antidiuretic hormone (ADH), since it disappeared after the induction of experimental diabetes insipidus No antidiuresis was obtained by infusions of hypertonic saccharose into the 3rd ventricle The activation of heat dissipation mechanisms by local warming of the preoptic/anterior hypothalamic region was not associated with any inhibition of the water diuresis However, profound local cooling of this part of the brain induced a release of ADH in hydrated animals The possibility is discussed, that this effect of local brain cooling may be due to a reduction of a neural inhibition, normally exerted on the ADH release

A hypothalamic 'osmoreceptor' mechanism appears to regulate the release of ADH from the neurohypophysis (Verney 1947 Jewell and Verney 1957) and may participate in the regulation of water intake (*cf* Andersson 1966) Variations in volume and distribution of the blood and the extracellular fluid without changes in tonicity, have also been found to modify the secretion of ADH This indicates that a "volumetric" regulation of the ADH release operates in parallel with the "osmometric" regulation (*cf* Gauer and Henry 1963) It is further known that several anesthetics increase the secretion of ADH which makes it impossible to induce a water diuresis by hydration in animals narcotized with these drugs (*cf* Walker 1957) With this in mind, Walker (1957) has put forward the hypothesis that the normal secretion of ADH also to a certain extent is regulated by neural inhibition of the supraoptico-neurohypophyseal system and that this inhibitory tonus is removed during the anesthesia Observations made in animals having the hypothalamus completely separated from the remainder of the brain support Walker's hypothesis (Suda, Koizumi and Brooks 1963) An increased spontaneous electrical activity of the supra-

optico hypophyseal tract is found in these animals which may indicate a loss of normal neural inhibitory regulation of the ADH secretion

Infusions of hypertonic solutions into the 3rd brain ventricle of hydrated go were recently performed to investigate the osmometric regulation of the AI secretion (Andersson Olsson and Warner 1967 Andersson Dallman and Ols 1969) The present work is an extension of these infusion studies supplemented w experiments performed to study the influence of preoptic warming and cooling the secretion of ADH The latter experiments seem to provide additional support Walker s theory of a neural inhibitory regulation of the ADH secretion

Methods

A nals Ter ad lt female goats (b w 30—40 kg) were sed The an nals ere rout conf ned in metal ol sm cages b means of collars and all experime s vere conducted tl cages The experimental periods for the ind dual goats var ed between 2 and 6 mon hs T m n mum nterval bet een the exper ments performed in each s ngle goat was 3 days T an mals had free access to chaffed hay and water and were fed al out 400 g of comme c gra n mix (with 6 g of NaCl added) each afternoon Room temperature was ma nta ne l $20 \pm 1°$ C

Implantat on of ventr cular ca ulae and infus on tech que S x of the goats (C 1—C ere prepared v tl permanent cannulae n tl e anter or part of tl e 3rd bra n ventr cle C of the goats C I lad a second permanent cannula placed n tl e posterior part of tl e 3 ventr le near the entrance of tl e mesencephal c canal To r m m ze tl e dea l space I ntraventr cular nfusions v ere performed v a an nner cannula wl cl was f lle l w tl the so t on to be nfu ed and wh ch was nserted to tl e ventr cular end of tl e permane t cann befo e an inf on as started The rate of nfu on was ma nta ne l e tl er at 7 5 10 o µl/m n An nfu on rate of 10 µl/m n was u ed in most exper ments The operat on and fus on tecl n ques l a e been descr bed in deta l earl er (Andersson *et al* 1967) In all infu exper ments cerebrosp nal flu d (CFS) as observed to dra n o t of tl e o ter [illegible] cannula on compress on of tl e neck botl l efore in ert on of an l after removal of tl e i r cannula sed for he nfus on Tl us a completely free m x ng of tl e infuse l solut on w l CFS of tl e 3rd en r cle as guaranteed n all tl e present exper m ents

The mode mplantat on and local b a n cool ng o ua n g Four of tl e goats T I T l ad a g lded s l er tl ermode mplanted med ally n tl e preopt c/an e or l yf otl alan c reg T 1—I 3 or n tl e forebra n cover ng only tl e anter or part of tl e preopt c reg on I F g 1 and 4 Local cool ng or warm ng of tl e sur ound ng b a n t s e was ol ta d perfus on of tl e tl ermodes w tl cold or arm ate The construct on of tl e tl e r o l m etl ods of i n pla tat on and tl e perfus on tecl n que e essent ally tl e same as earl descr bed Andersson *et al* 1963 The degree of central cool ng or warm ng was mea l a needle appl cator for temperat e record ng l a ng ts tl ermo ens t ve t p rest ng o dorsal edge of l e therm de chamber l etween the n and ou flo annulae F g 1

Induct on of expe mental d abet s ns p du Two of tl e can lae goats C 2 C 3 a one of tl e thermode an mals T I were al o pro de l l a pa r of tl ermocou le e e l ha ng tl e r un ns lated t ps 3 m m placed l laterally n the med an em nence (F g 1 one stage du g tl e exper mental per od these electro les were used to produce a ra io-f quenc les on n tl e med an em nen e accord ng to tl e te hn q e described l y Gale 1 3 Rad o-frequen heat ng of tl e med an en nen e to 70 C for 5 m n was perform ed w l o anes hes a an l witl tl e — — No nd cat on of [illegible] d stress was seen du g al onnect o b tween tl e l pothalamus occurre ce [illegible] tl e tran ent and tl e pe s n tl e tl ree go t

Hyd at on and u n ng tl e goats also

100 ml of tep d wa er kg b w l o na h tube one l r before ur ne col ect on was starte l l des red tl e h dra on uld l e ma n a ned for lo g per ods of t me by g ng an ad l t on 00 ml of wa er at l u l n erva Tl e ur ne wa colle ted n 10 or 0 m n sa n les via a retent n cathe er se ed nto tl e u nary l la lder

Analyse Ur nary Na a d K o ce trat o were determ ned u ng an EEL flame pl ot meter and tl e Cl on en ra o wa de erm ned by tl e m etl od of B un 1949 For determ na t ons of ur ne o molar ty a Knaue osmome er was u ed



Fig 1 An X ray picture (lateral view) of the skull of goat T 1 To the left is seen the thermode implanted medially in the preoptic/anterior hypothalamic region The needle applicator for recording of the thermode temperature is placed between the in and outlet cannulae of the thermode chamber Two parallel thermocouple electrodes (covering each other in the picture) are seen to the right of the thermode The 3 mm uninsulated tips of these electrodes are located bilaterally in the median eminence Diabetes insipidus was induced by radio-frequency heating between the electrode tips

Results

A *Intraventricular infusions*

It was previously shown (Andersson *et al* 1967) that brief infusions of hypertonic NaCl and NH_4Cl into the anterior part of the 3rd ventricle caused a temporary inhibition of the water diuresis of hydrated goats The antidiuretic effect of NH_4Cl was greater than that of NaCl No such inhibition was obtained by similar infusions of hypertonic d glucose However while the intraventricular infusions of hypertonic NaCl effectively elicited drinking in the non hydrated goat similar infusions of hyper tonic NH_4Cl or d glucose did not induce any urge to drink The apparent dissimilarity between central regulation of water intake and of ADH release led to the following analysis of the antidiuretic effect of infusions of NH_4Cl into the 3rd ventricle Furthermore the lack of antidiuretic and drinking effects of hypertonic d glucose made it of interest to study whether infusions of hypertonic saccharose into the 3rd ventricle might elicit any of these effects

Ammonium chloride

The two-cannula goat (C 1) was used in an attempt to delimit the region of the brain mediating the antidiuretic effect of the NH_4Cl infusions Repeated brief (3 min) infusions of 0 4 M NH_4Cl were made alternatively via the front and via the back cannula of this animal during hydration Infusions made into the anterior part of the 3rd ventricle (in front of the massa intermedia) elicited a more pronounced inhibition of the water diuresis than did identical infusions made into the posterior

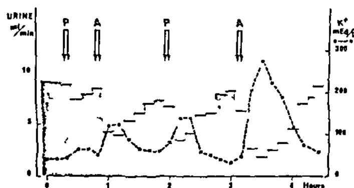


Fig 2 An experiment in the two cannula goat C 1 during hydration demonstrating the stronger antidiuretic response to brief (5 min) infusions of NH_4Cl (0 4 M) into the *anterior part of the 3rd* ventricle (A) as compared to similar infusions made in the posterior part of the ventricle (P) near the entrance of the mesencephalic canal The first two infusions were made at a rate of 7 5 µl/min the following two at 15 µl/min

part of the ventricle (behind the massa intermedia near the entrance of the mesencephalic canal) (Fig 2)

Direct evidence that the NH_4Cl induced inhibition of the water diuresis was due to a release of ADH from the neurohypophysis was obtained in goats C 2 and C 3 During the month preceding radio frequency lesioning of the median eminence of these animals brief (2—3 min) infusions (7 5 µl/min) of hypertonic (0 85 M) NH_4Cl were made several times into the anterior part of the 3rd ventricle during hydration These infusions invariably caused a temporary inhibition of the water diuresis concomitant with a rise in urinary electrolyte concentration However after the lesioning of the median eminence during the transitory and the permanent stages of diabetes insipidus even prolonged (10 min) intraventricular infusions of 0 85 M NH_4Cl solution did not cause any significant decrease in urine flow or any rise in urinary electrolyte concentration

Intraventricular infusions of solutions practically isotonic to the CSF indicated *that the NH_4Cl induced antidiuresis was not due to a physiological stimulation of* a hypothalamic osmoreceptor mechanism Brief (4 and 5 min) infusions (10 µl/min) of 0 15 M NH_4Cl were made into the anterior part of the 3rd ventricle of three of the goats (C 4 C 5 and C 6) during hydration Similar infusions of equimolar NaCl were used as control In contrast to the NaCl infusions the infusions of isotonic NH_4Cl resulted in a pronounced temporary inhibition of the water diuresis and a simultaneous rise in urine osmolarity (Fig 3 left)

Saccharose

Three goats (C 4 C 5 and C 6) were subjected to 10 min infusions (10 µl/min of 1 M saccharose into the 3rd ventricle during hydration For comparison brief (5 min) intraventricular infusions of 0 5 M NaCl were also made at the same rate during each hydration period The infusions of hypertonic NaCl caused a pronounced inhibition of the water diuresis and a conspicuous rise in urine osmolarity However in spite of the larger osmotic load delivered into the 3rd ventricle during the saccharose infusions these infusions did not diminish the water diuresis or cause any increase in urine osmolarity (Fig 3 right)

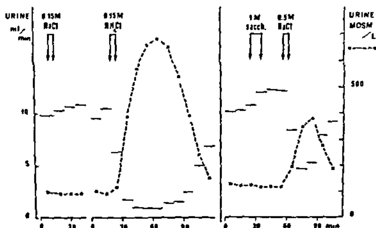


Fig 3 *Left* Pronounced inhibition of the water diuresis and a concomittant rise in the urin osmolarity obtained by a brief (5 min) infusion (10 μl/min) of isotonic NH_4Cl into the ar terior part of the 3rd brain ventricle of goat C 4 For comparison is shown the lack of effec of a similar infusion of isotonic NaCl made during the same hydration period

Right Lack of antidiuretic effect of a 10 min infusion (10 μl/min) of 1 M saccharose int the 3rd ventricle of goat C 5 during hydration An antidiuretic response is obtained by a sul sequent 5 min infusion (10 μl/min) of 0 5 M NaCl, in spite of the smaller osmotic loa delivered into the ventricle during this infusion

In two preliminary experiments 1 M saccharose was infused (10 μl/min) into th anterior part of the 3rd ventricle for 30 min in the non hydrated goat These infu sions did not elicit drinking while control infusions of NaCl solution of the sam tonicity (0 5 M) caused the expected cumulative drinking of large amounts of water Further experiments are needed however to prove that the infusion of hypertoni saccharose into the 3rd ventricle does not stimulate the thirst mechanism

B Local brain cooling

Affected brain regions

In three of the goats (T 1—T 3) the thermode placement was almost identical Due to the relatively large lateral surface of the medially placed thermode, the tempera ture of the preoptic region the dorsal part of the anterior hypothalamus and the ventral septum must have been lowered in these goats during cooling (Fig 4) Since the temperature gradient is very steep close to the thermode during brain cooling with this technique (Andersson *et al* 1963) it can be assumed that primarily the medial parts of these brain regions were exposed to cooling A fall in temperature as far distant as in the supraoptico-hypophyseal tract apparently did not occur since measurements performed in goat T 1 revealed that median eminence temperature was not lowered during profound central cooling (thermode 25° C)

Due to the different thermode placement mainly the anterior division of the pre-optic region and more rostral parts of the fore-brain, were exposed to cooli goat T 4 (Fig 4)

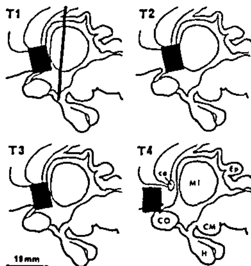


Fig 4 Placement of the thermode chambers in the four thermode goats used A lowering of the temperature of the thermodes of goats T 1—T 3 by 8° C or more caused an inhibition of the water diuresis of the goats which by all probability was due to a release of ADH No such response was obtained in goat T 4 having the diverging thermode placement The position of the thermocouple electrodes used to induce diabetes insipidus in goat T 1 is also indicated in the figure
ca = Anterior commissure
CM = Corpus mammillare
CO = Chiasma opticum
Ep = Epiphysis
H = Hypophysis
MI = Massa intermedia
Light shaded area marks the extension of the 3rd brain ventricle

Influence on ADH release

A lowering of the thermode temperature by more than 8° C below normal brain temperature for periods of 5 min or longer, caused a temporary inhibition of the water diuresis and a rise in urine osmolarity of goats T 1—T 3 None of these effects was seen in goat T 4 even when the thermode temperature was lowered to 15° C

An analysis of the antidiuretic response to central cooling in goats T 1—T 3 revealed that the degree of antidiuresis was related to the intensity of the local brain cooling (Fig 5 above) It was also found that the duration of the antidiuresis was related to the length of the cooling period (Fig 5 below)

Direct evidence that the observed inhibition of the water diuresis was due to a release of ADH from the neurohypophysis was obtained in goat T 1 During the transitory and permanent stages of diabetes insipidus induced by radio-frequency lesioning of the median eminence cooling of the preoptic/anterior hypothalamic region no longer reduced the urine flow or caused any rise in urine osmolarity (Fig 6)

There was no apparent correlation between the antidiuretic and the thermoregulatory responses to central cooling In all animals including goat T 4 (not responding with antidiuresis) the central cooling induced peripheral vasoconstriction and a gradual rise in rectal temperature During the first experiments made in goats T 2 and T 3 strong shivering occurred The shivering response to central cooling gradually diminished during the experimental period No simultaneous adaptation of the antidiuretic response was observed however

It has recently been shown that cooling restricted to the midpreoptic region may induce a brisk water diuresis in the *ad lib* hydrated rhesus monkey (Hayward and Baker 1968) For this reason the present goats were also subjected to prolonged (1

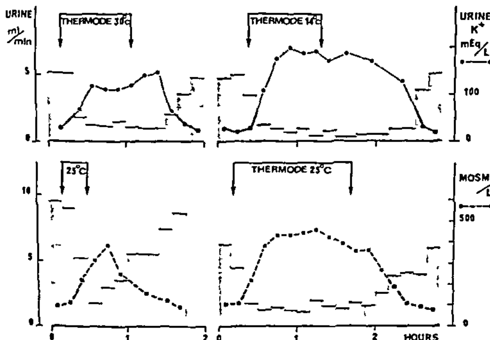


Fig 5 *Above* Two experiments performed in goat T 2 during hydration which demonstrate that the degree of antidiuresis is dependent of the intensity of the cooling of the preoptic/anterior hypothalamic region

Below Two experiments in goat T 3 during hydration showing that the duration of the antidiuresis obtained by preoptic/anterior hypothalamic cooling is related to the length of the cooling period

to 2 hr) central cooling (thermode temperature 25 or 30° C) when not pre-hydrated No water diuresis developed in these experiments

C Local brain warming

The thermodes of goats T 1 and T 3 were also used for local warming of the surrounding brain tissue during hydration In these experiments the thermode temperature was maintained at 41° C for periods of 30 to 60 min As expected from previous work (Magoun *et al* 1938, and others) the preoptic warming induced polypnea, peripheral vasodilatation and a gradual fall in rectal temperature However, the water diuresis and the urine osmolarity were not changed by the central warming

Discussion

According to Verney (1947) the hypothalamic 'osmoreceptors' are not stimulated by a rise in body fluid tonicity *per se* but rather by changes in the extracellular fluid which reduces the volume of the receptors This concept is based on the finding that intracarotid injections of hypertonic sodium salts saccharose and glucose cause a release of ADH in hydrated dogs while similar injections of hypertonic urea are inef-

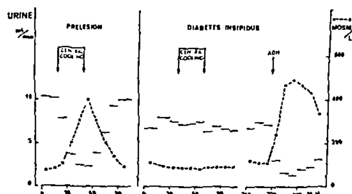


Fig 6 *Left* The antidiuretic response to local cooling (thermode 25° C) of the preoptic/anterior hypothalamic region of goat T 1 before experimental diabetes insipidus was induced in this animal

Right Complete lack of response to identical cooling during the transient phase of diabetes insipidus, *indicating that the prelesion effect of the central* cooling was due to a release of ADH from the neurohypophysis A normal antidiuretic response was obtained by a subsequent intravenous injection (*ADH*) of 6 mU of antidiuretic hormone (Vasopressin "Sandoz")

fective in this respect Verney explained the lack of response to intracarotid injections of urea by the high diffusibility of this substance This classical picture of the function of the "osmoreceptors" appears to need revision in the light of later findings that urea passes the blood-brain barrier very slowly, and that intravascular injections of hypertonic urea cause a marked dehydration of the central nervous system (*cf* Kleeman and Cutler 1963)

It was previously found that slow infusions of hypertonic NaCl into the anterior part of the 3rd ventricle stimulate the release of ADH in the hydrated goat and elicit thirst in the non-hydrated animal (Andersson *et al* 1967 and 1969) Both effects may be explained as due to a stimulation of 'osmoreceptors" involved not only in the regulation of the ADH release but also in the regulation of water intake On the other hand, the previous experiments in the goat revealed that intraventricular infusions of hypertonic NH_4Cl had a powerful antidiuretic effect, but did not elicit any urge to drink in the non-hydrated animal This appeared incompatible with the concept of an "osmoreceptor" mechanism having the dual function mentioned above The present study provides direct evidence that the antidiuresis occurring when NH_4Cl is applied in the 3rd ventricle is due to a release of ADH, and indicates that the anterior part of the diencephalon mediates the effect Furthermore, the use of isotonic NH_4Cl solutions has revealed that the antidiuretic effect of NH_4Cl remains in the absence of 'osmotic' stimulation

Ammonium chloride has been found to stimulate both normal and denervated ganglia (Banister Hebb and Konzett 1949) A stimulation of the cells of the cerebral cortex is also obtained by local application of NH_4 ions in low concentration while higher concentrations of NH_4 inhibits the activity of such cells (Gore and McIlwain 1952) Since in the present experiments < 10 *µ*mol of NH_4Cl infused into the anterior part of the 3rd ventricle elicited antidiuresis (Fig 3 left) it seems more likely that the antidiuresis was the result of a stimulatory rather than of an inhibitory effect of

the NH_4 ions If so the NH_4 stimulation appears much more selective than the non specific stimulation obtained by K, since infusions of KCl into the 3rd ventricle elicit several effects not seen during corresponding NH_4Cl infusions (*e g* thirst and natriuresis) (Olsson 1969)

The concept of an osmometric regulation of the ADH release is to a great extent founded on the observation that intracarotid infusions of hypertonic saccharose stimulates the release of ADH effectively in the hydrated dog (Verney 1947) That no antidiuretic or drinking response to intraventricular infusions of hypertonic saccharose occurred in the present goats does not necessarily contradict the existence of an osmometric regulation in this species In their studies of the properties of the cerebrospinal fluid system in the unanesthetized goat Pappenheimer and co-workers (Pappenheimer *et al* 1962 Heisey Held and Pappenheimer 1962) have found that fructose infused into the ventricular system diffuses very slowly through the ependyma into the brain tissue and that the intact ventricular wall is practically impermeable to inulin It must be assumed therefore that very little of the saccharose infused into the 3rd ventricle of the present goats may have penetrated into the hypothalamus Under such circumstances however the saccharose induced hypertonicity of the CSF of the 3rd ventricle ought to have caused a passage of water in the opposite direction It remains to be explained why this dehydration of adjacent parts of the brain did not act as a stimulus to an osmoreceptor mechanism

Thermoregulatory mechanisms are known to influence body fluid homeostasis and the cold diuresis in man has been attributed to an inhibition of the release of ADH from the neurohypophysis (*cf* Bass and Henschel 1956) That a change in the activity of the preoptic thermoregulatory center may be involved in a cold induced block of the ADH release is evidenced by the observation that midpreoptic cooling in the rhesus monkey may induce a water diuresis which can be inhibited by the administration of exogenous ADH (Hayward and Baker 1968) Since preoptic cooling initiates a thermoregulatory shift of blood from the periphery to the central veins this diuresis has been attributed to a reflex volumetric inhibition of the ADH secretion It can not be excluded that cooling involving only the midpreoptic region may induce a water diuresis also in the goat However the local cooling of a wider area of the brain including not only the preoptic region but also parts of the anterior hypothalamus and the ventral septum did not elicit any water diuresis in the non hydrated goat In contrast this cooling inhibited the water diuresis of hydrated animals The experiments performed before and after diabetes insipidus had been induced in goat T 1 showed that the inhibition of the water diuresis by all probability was due to a release of ADH This release of ADH was apparently not related to the intensity of the thermoregulatory response of the goats and was not elicited when only the anterior part of the preoptic region and adjacent parts of the fore brain were affected by the cooling (goat T 4 That a rather profound cooling was needed to elicit the antidiuresis gives a further indication that the response was not mediated by a stimulation of central cold receptors

Of the hypothalamic neurosecretor cells primarily those in the paraventricular

nuclei were affected by the cooling The hypothermia may have induced changes in the membrane permeability of these cells However, a leakage of antidiuretic material out of the neurosecretory cells during the central cooling seems unlikely, since the antidiuretic response to cooling had already disappeared during the transient phase of the diabetes insipidus, i e at a stage when neurosecretory material must still have been present in the paraventricular cells As mentioned in the introduction it has been suggested that a neural inhibition of the supraoptico neurohypophyseal system may be an essential link in the normal regulation of the ADH secretion The possibility remains that the local brain cooling performed in the present goats may have diminished an inhibitory tonus of this kind This would explain why the degree of antidiuresis was dependent of the intensity of the local brain cooling and why its duration was related to the length of the cooling period

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Histochemical Characterization of a Tryptamine-Like *Substance Stored in Cells of the Mammalian Adenohypophysis*

By

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Abstract

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[illegible]

pituitary fluorophore, moreover, showed the same microspectrofluorimetric characteristics under various conditions as does the tryptamine fluorophore. These histochemical and microspectrofluorimetric characteristics differ clearly from those exhibited by a number of catechol and indole compounds, including the biogenic catecholamines, 5-hydroxytryptamine and tryptophan. The reliability and the significance of the histochemical criteria are discussed, and it is concluded that there is much to support the view that tryptamine, or a closely related β(3-indolyl)ethylamine, is stored in the adenohypophysis. Another fluorophore that may derive from a hitherto unknown biogenic phenylethylamine was disclosed in a large number of pars distalis cells not identical with those that possibly store tryptamine.

Cells displaying specific formaldehyde-induced fluorescence in the Falck—Hillarp fluorescence procedure have been described in the adenohypophysis of mammals (Dahlström and Fuxe 1966, Björklund, Falck and Rosengren 1967, Björklund, Enemar and Falck 1968). The nature of the fluorigenic substance stored in these cells was dealt with in a recent report (Björklund and Falck 1969). This microspectrofluorimetric and chemical investigation of pituitary glands from normal and reserpine-treated cats showed that the substance was not identical with the biogenic catecholamines (dopamine, noradrenaline and adrenaline) or 5-HT. Moreover, preliminary results suggested that it could be identical with tryptamine. To evaluate this hypothesis, the possibilities for the histochemical demonstration of tryptamine and some related indole compounds by means of formaldehyde-condensation techniques have been studied (Björklund, Falck and Håkanson 1968). It was found that

formaldehyde treatment, in combination with treatment with a gaseous oxidant (ozone) or acidification of tissue sections after the conventional formaldehyde treatment, dramatically increased the fluorescence yield of tryptamine, whereas that of the biogenic catecholamines and 5 HT was strongly reduced upon the combined formaldehyde-ozone treatment There are reasons for believing that these procedures provide possibilities for a sensitive and almost selective histochemical demonstration of tryptamine at the cellular level, furthermore, the studies of the microspectrofluorimetric characteristics of tryptamine and some related amines and amino acids in models under the various histochemical conditions offered good possibilities for the microspectrofluorimetric identification of tissue tryptamine (see Bjorklund Falck and Håkanson 1968) In the present investigation, these histochemical procedures were applied to the adenohypophysis of the rat, cat, and pig in combination with microspectrofluorimetric analyses of the formaldehyde-induced fluorophores It was found that the fluorigenic substance in certain adenohypophyseal cells of all three species behaved like tryptamine in models, which supports the view that tryptamine is stored normally in these cells

Material and methods

Animals Pituitary glands from 30 rats (Sprague Dawley), 12 cats, and 26 pigs of both sexes were used The rats weighed 150—300 g they were killed by decapitation under ether anesthesia The cats which weighed more than 0 8 kg were killed by bleeding under pentobarbital sodium anesthesia 6 pigs (about 3 months old and 20 kg in weight) were killed at the laboratory by bleeding under pentobarbital sodium anesthesia The remaining 20 pig pituitary glands were obtained at a slaughter house from appr 5 months old fattened pigs

Histochemical procedures and fluorescence microscopy All the pituitary glands were excised within 5 min after killing and immediately quenched in liquid propane at the temperature of liquid nitrogen except the slaughter house material, which was frozen within 20—25 min after death After freeze drying the specimens were treated with gaseous formaldehyde and/or ozone in a closed vessel (1000 ml) at +80° C according to Bjorklund Falck and Håkanson (1968) Ozone was formed at room temperature from the oxygen of the air inside the closed reaction vessel by means of an electric discharge between two electrodes generated by a Tesla coil with the specimens and the paraformaldehyde [illegible] vessel was varied by varying the time of the e[illegible] dehyde was generated from paraformaldehy[illegible] humidity thus containing about 0 6 % water

Five types of treatments were performed (1) the specimens were exposed to formaldehyde gas in normal air at +80 C for 1 hr (i e conventional formaldehyde treatment cf Falck and Owman 1965) 2 the specimens were exposed to formaldehyde gas in ozone enriched air at +80° C for 1 hr 3) the specimens were first exposed to formaldehyde gas in normal air at +80° C for 1 hr and then transferred to another vessel without formaldehyde in which ozone was generated The specimens were then kept in this ozone-enriched air at +80 C for 1 hr (4) the two steps under 3 were performed in reverse order (5) the specimens were exposed to ozone-enriched air without formaldehyde at +80° C for 1 hr Specimens exposed neither to formaldehyde nor to ozone were used as controls To save material the cat pituitary glands were usually divided by a median sagittal section and the two halves were treated differently

In order to compare the relative yield of the formaldehyde induced fluorescence after the type 1 and type 2 treatments the pituitary glands from 2 rats 2 cats and 3 pigs (from the slaughter house were divided by a median sagittal section One half of each gland was treated with formaldehyde in normal air type 1 treatment and the other half with formaldehyde in ozone-enriched air (produced by a 20 min discharge in the vessel) i e type 2 treatment In this series the formaldehyde was generated from paraformaldehyde equilibrated in air with c.a 30 % relative humidity about 0 3 % water content cf Hamberger 1967) This comparatively low humidity of the paraformaldehyde was chosen since pilot experiments suggested that

higher humidity increased the risk of concentration quenching of the fluorescence in the cat pars intermedia, which exhibited the strongest specific fluorescence (*cf* Results) The two halves from the same gland were blocked together in paraffin so that the recordings of the [illegible] ts could be compared in the same [illegible]

[illegible] ce microscopy according to Falck [illegible] Falck (1968) [illegible] ns from glands from the three [illegible] al air (type 1 treatment) or in ozone enriched air (type 2 treatment, ozone concentration corresponding to 20 min discharge) were fastened to quartz or glass microscope slides with a thin layer of albumin glycerin and were then deparaffinized in xylene for 5 min The sections were exposed to the vapours from concentrated HCl and NH_3 solutions at room temperature for 30 sec—10 min and then mounted in xylene Other sections were deparaffinized in xylene and then mounted in glacial acetic acid and examined after 5—20 min Sections from glands not treated with formaldehyde or ozone were used as controls

In order to compare the fluorescence picture on microphotographs of one and the same section after the various treatments sections from glands exposed to formaldehyde gas in normal air (type 1 treatment *cf* above) were glued to microscope slides with a thin layer of album [illegible]

Microspectrofluorimetric analyses The recordings of the fluorescence emission and excitation spectra were performed on sections mounted in xylene on quartz microscope slides (Suprasil I®) with a modified Leitz microspectrograph The procedure has been described elsewhere (Bjorklund Ehinger and Falck 1968 Bjorklund and Falck 1969), only one alteration of the instrument was introduced in the present investigation the original prism monochromator for the exciting light was replaced by a Zeiss M 20 grating monochromator All spectra were corrected according to the procedures described earlier (Ritzen 1967, Bjorklund Ehinger and Falck 1968) and the spectra are expressed as relative quanta versus wavelength The recordings of fluorescence intensity were performed according to Bjorklund Falck and Håkanson (1968) on sections containing tissue from two differently treated halves of the same gland (*cf* above) A constant area covering appr one intermedia cell was measured in the pars intermedia 30 recordings from each half of the gland were made in the pars intermedia on 6—10 sections and the mean values ± S E M were calculated Non fluorescent areas of the neural lobe were measured in the same way to obtain blank values In this way the relative yield of formaldehyde induced fluorescence in the intermedia cells after different treatments could be estimated Because of the errors of this recording procedure as well as the difficulties of standardizing the histochemical procedures the intensity values must be regarded as semi quantitative (*cf* Bjorklund Falck and Hakanson 1968)

Results

Fluorescence microscopy

A Conventional formaldehyde treatment After treatment with formaldehyde gas at +80° C for 1 hr nearly all cells in the *pars intermedia* of the rat, the cat and the 5 month old pigs showed a yellow formaldehyde induced *i e* specific fluorescence with a cytoplasmatic localization (Fig 1c and 2a) whereas no obvious fluorescence was seen in the pars intermedia of the 3 month old pigs The fluorescence showed a clear fading upon UV irradiation and was quenched when the section was mounted in water, which is characteristic for specific monoamine fluorescence (Falck and Owman 1965 Corrodi and Jonsson 1967) The fluorescence intensity was usually rather constant throughout every single lobe but showed remarkable inter individual varia

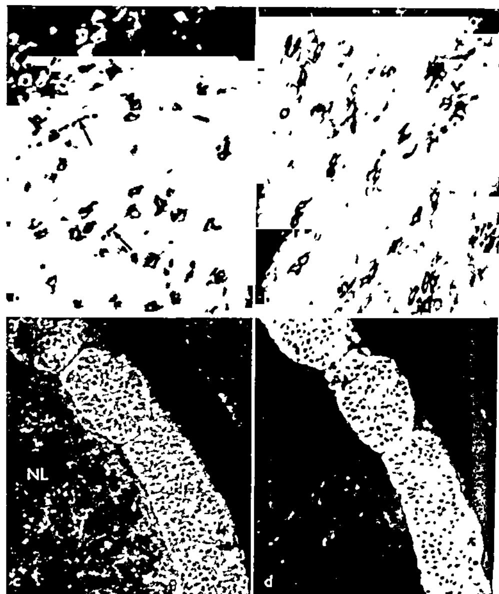


Fig. 3. Cells in the pituitary gland of the rat displaying the fluorescence characteristic for tryptamine. a) Pars distalis after conventional formaldehyde treatment (type 1 treatment, cf. Material and Methods). The section was mounted in glacial acetic acid. The cells showed no specific fluorescence before the acidification. The reddish, unspecific fluorescence evoked upon mounting in the acid is seen within some vessels (arrows). (×240). b) Pars distalis after formaldehyde treatment in ozone-enriched air (type 2 treatment). (×150). c) Pars intermedia (PI) and neural lobe (NL) after conventional formaldehyde treatment. A low specific fluorescence is seen in the intermedia cells, and a plexus of delicate varicose fibres in both lobes exhibit a fluorescence characteristic for catecholamines. (×180). d) The same section as in c) after a subsequent exposure to formaldehyde in ozone-enriched air (type 2 treatment). The intensity of the cellular fluorescence is strongly increased, whereas the catecholamine fluorescence in the fibres has disappeared. (×180).

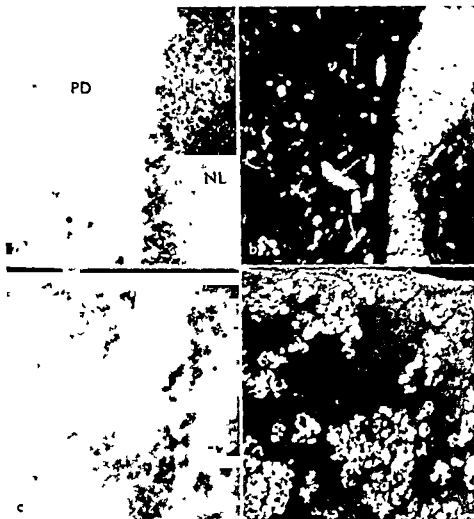


Fig 2 Yellow fluorescence characteristic for tryptamine in the cat pituitary a) and c) After conventional formaldehyde treatment b) The same section as in a) after acidification by mounting in glacial acetic acid A strong increase in the fluorescence intensity in cells of the pars intermedia (PI) and pars distalis (PD) is seen upon the acidification NL=neural lobe The unspecific reddish fluorescence s seen within vessels (arrows) d) The same section as in c) after a subsequent exposure to formaldehyde in ozone-enriched air (type 2 treatment The weakly fluorescent cells in c show a striking increase in fluorescence intensity after the ozone treatment × 180)

tions from sometimes barely visible to strong among the cats and among the 5 month old pigs In the pars intermedia of the rat the inter individual variation was much less pronounced and the fluorescence weak and often barely visible However previous results have demonstrated markedly stronger fluorescence in the rat pars intermedia and preliminary observations suggest that a periodical possibly seasonal variation in the concentration of the fluorigenic substance occurs in this species The rat pituitaries used in this study were obtained during October to December

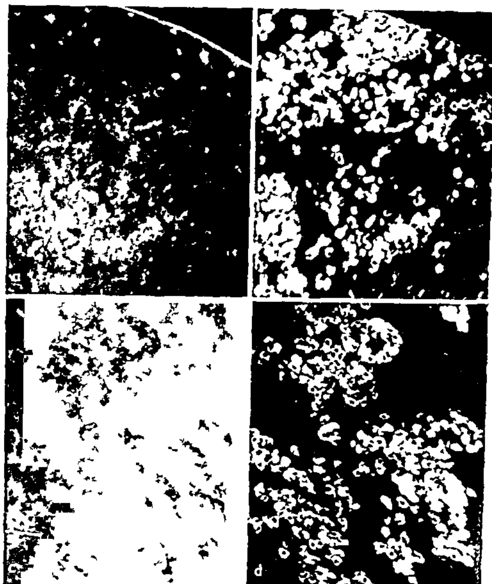


Fig 3 Yellow fluorescence characteristic for tryptamine in the pars distalis of the p g a and c) After conventional formaldehyde treatment b The same section as in a when mounted in glacial acetic acid d The same section as in c) after a subsequent treatment with formaldehyde in ozone-enriched air type ? treatment The very low specific fluorescence in the pars distalis cells after the conventional formaldehyde treatment shows a striking increase in intensity *after the acidification and after the ozone treatment* ×180

A small proportion of the cells in the pars intermedia showed a moderate to strong fluorescence which was blue with the filter setting used in this study (With the commonly used filter setting i e BG12 as primary and Zeiss 47 + 50 as secondary filters the colour was green These cells were most common in pars intermedia of the 5 month old pigs and rare in the cat and rat Some few intermedia cells with no

detectable specific fluorescence were usually seen scattered throughout the lobe in all the animals

Besides these cells there appeared a rich system of fluorescent catecholamine containing fibres in the pars intermedia and the neural lobe as reported in detail earlier (Bjorklund Falck and Rosengren 1967, Bjorklund 1968 Bjorklund and Falck 1969)

In the *pars distalis*, cells with a yellow formaldehyde induced fluorescence were seen in the cat and the 3 and 5 month old pigs but not in the rat after the conventional formaldehyde treatment The fluorescent pars distalis cells of the cat showed a clearly stronger fluorescence than those of the pig which displayed a weak to sometimes barely visible fluorescence (Fig 2a c and 3a c) The cells occur in a large number throughout the lobe The fluorescence showed fading upon UV irradiation and quenching upon mounting in water similar to the intermedia cell fluorescence

B Acidification after conventional formaldehyde treatment Sections obtained from the glands treated with formaldehyde gas at +80° C for 1 hr were exposed to HCl vapour or mounted in glacial acetic acid (*cf* Material and Methods) The acidification caused a dramatic increase in the intensity of the yellow specific fluorescence of the cells in the pars intermedia and pars distalis of all three species (Fig 1 2 and 3) A large number of cells in the pars distalis of the rat and most of the intermedia cells in the 3 month old pigs which were non fluorescent after the conventional formaldehyde treatment displayed a moderate to strong fluorescence after acidification (Fig 1a) The yellow fluorescent cells were the only fluorescent cells seen in the pars distalis after a short exposure to HCl vapour or when the sections were mounted in glacial acetic acid After longer HCl treatment a second population of fluorescent cells appeared in the pars distalis of the rat and the 3 and 5 month old pigs These cells which occurred throughout the lobe exhibited a fluorescence of moderate intensity the emitted light being blue or green according to the filter setting used as was the case with a few intermedia cells (see above) This pH dependent fluorescence will be dealt with in a separate investigation The intensity of the general low and non specific fluorescence in the structures outside the specifically fluorescent cells was unaffected or decreased slightly upon acidification

In the control specimens not treated with formaldehyde a very low general background fluorescence was seen (*cf* Bjorklund and Falck 1969) This picture remained unchanged upon the various types of acidification except that mounting in glacial acetic acid induced for unknown reasons a reddish fluorescence within blood vessels This unspecific fluorescence was seen also in the formaldehyde treated specimens arrows in Fig 1a and 2b)

C Formaldehyde ozone treatments After formaldehyde treatment in ozone-enriched air (type 2 ozone + formaldehyde gas 1 hr +80° C) a fluorescence yield was obtained in the yellow fluorescent cells in the pars intermedia and pars distalis which was much higher than that obtained after conventional formaldehyde treatment

TABLE I Relative fluorescence yield of the yellow fluorescent intermedia cells of the rat, cat and pig after two different treatments (1) conventional formaldehyde treatment i e formaldehyde treatment in normal air for 1 hr at +80° C, (2) formaldehyde treatment in ozone-enriched air for 1 hr at +80° C (the ozone concentration corresponded to 20 min electric discharge *in the reaction vessel)* Each pair of recordings were peformed on the two halves of the same gland (*cf* Material and Methods) The intensity is expressed with the intensity value after the conventional treatment as unit S E M is given within brackets

Animal	Formaldehyde (1)	Formaldehyde+ozone (2)
rat I	1.00 (0.15)	12.79 (1.37)
rat II	1.00 (0.12)	14.83 (1.57)
cat I	1.00 (0.05)	8.76 (0.57)
cat II	1.00 (0.05)	14.57 (0.66)
pig I	1.00 (0.07)	18.61 (1.49)
pig II	1.00 (0.09)	16.71 (1.19)
pig III	1.00 (0.14)	10.01 (0.65)
MV	*1.00*	*13.67*

(type 1 formaldehyde gas 1 hr, +80° C) (Fig 1, 2 3 and Table I) In the pars distalis of the rat and in the pars intermedia of the 3 month old pigs where no fluorescent cells were seen after formaldehyde treatment alone, a rich amount of yellow fluorescent cells with a moderate to strong intensity appeared after the formaldehyde ozone treatment (Fig 1b), the picture being quite similar to that obtained upon acidification of the sections after conventional formaldehyde treatment (Fig 1a and b) The increase in the fluorescence yield was maximal at an ozone concentration obtained after about 20 min electric discharge The fluorescence showed a marked fading a upon UV irradiation and only a low fluorescence was seen after mounting in water No blue fluorescent cells were seen after the combined formaldehyde ozone treatment not even after subsequent acidification of the sections

A comparison of the relative fluorescence yield in the intermedia cells after formaldehyde treatment alone and after optimal formaldehyde ozone treatment according to Table I showed that the latter treatment produced a 9—19 times higher fluorescence intensity the average increase in the fluorescence yield of the seven recordings being 14 times The magnitude of this increase is very similar to and also characteristic for tryptamine (Bjorklund Falck and Håkanson 1968)

A similar increase in the fluorescence yield of the cells in the pars intermedia and pars distalis was obtained when the ozone treatment was performed after the formaldehyde treatment (type 3 formaldehyde gas 1 hr +80° C followed by ozone 1 hr +80 C) but in this case a maximal fluorescence yield was achieved at an ozone concentration corresponding to 45 60 min discharge When the ozone treatment (more than 20 min discharge preceded the formaldehyde treatment (type 4 ozone 1 hr 80 C followed by formaldehyde gas 1 hr +80 C) a clear decrease in the fluorescence yield was observed compared with the conventional formaldehyde treat

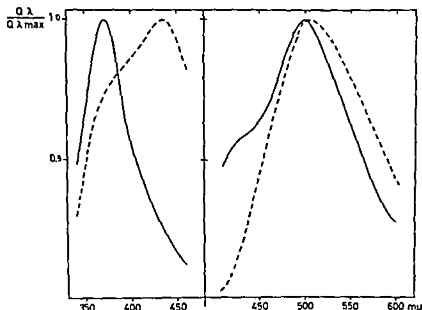


Fig 4 pH dependent variations in the excitation and emission spectra of the yellow fluorescent intermedia cells of a 5 month old pig after conventional formaldehyde treatment Similar spectra were obtained from the fluorescent cells in the pars distalis of the pig and cat and in the pars intermedia of the rat and cat

(———) after formaldehyde treatment only
( ) after formaldehyde treatment and subsequent mounting in glacial acetic acid
( ) after formaldehyde treatment and subsequent exposure to NH_3 vapour at room temperature for 1 min All spectra are expressed as relative quanta versus wavelength

ment The decrease was only slight after 20 min discharge but pronounced after longer discharges No obvious increase in the low unspecific fluorescence in the various structures of the pituitary glands was seen when the specimens were exposed to ozone alone at +80° C for 1 hr The combined formaldehyde-ozone treatments—*i e* the type 2 and 3 treatments—caused no or only slight increase in the general background fluorescence in the structures outside the cells that showed specific fluorescence (*cf* Fig 1b d 2d and 3d)

Microspectrofluorimetric analyses

A Conventional formaldehyde treatment (1 hr +80° C) The microspectrofluorimetric characteristics of the formaldehyde induced fluorescence in the adenohypophysis of the cat after this treatment have been reported earlier (Bjorklund and Falck 1969 After the formaldehyde treatment the yellow fluorescent cells in the pars intermedia of the rat and the 5 month old pigs and in the pars distalis of the pig showed excitation/emission spectra with the maxima at 370/495 mμ (Fig 4) In the emission spectrum a shoulder was sometimes recorded at about 430 mμ The emission peak maxima of the tissue parts showing the low unspecific fluorescen

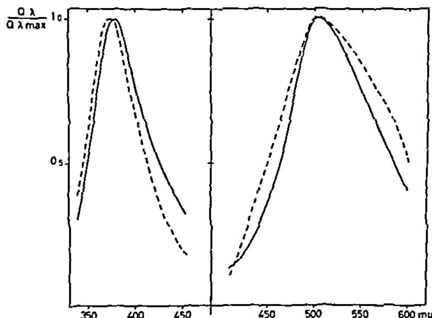


Fig 5 Excitation and emission spectra of the yellow fluorescent cells in the pars distalis of the rat No specific fluorescence appears in these cells after the conventional formaldehyde treatment

(———) treatment with formaldehyde in ozone-enriched air (type 2 treatment ozone concentration corresponding to 20 min electric discharge) at +80° C for 1 hr

(- -) after conventional formaldehyde treatment and subsequent mounting in glacial acetic acid

of the autofluorescence of not formaldehyde treated tissues had about the same position as this shoulder (*cf* Björklund and Falck 1969) and the height of the shoulder in the emission curves of the fluorescent cells was largely due to the choice of blank tissue for the spectral recordings However, irrespective of the choice of blank tissue a residual shoulder at 430 mμ was often recorded (Fig 4) and thus the presence of two peaks in the emission spectrum of the formaldehyde induced fluorescence cannot be excluded

Upon acidification of the sections after the formaldehyde treatment the emission maximum was unchanged or slightly displaced to longer wavelengths (500–510 mμ) (Fig 4) The shoulder at 430 mμ was usually no longer recorded In the excitation spectrum a second peak at about 430 mμ developed upon acidification and was often the dominating one after longer times of treatment in acid (Fig 4) As reported above the acidification caused a marked increase in the intensity of the specific fluorescence (*cf* Fig 2a b and 3a b) Also in the pars distalis of the rat and in the intermedia cells of the 3 month old pigs a similarly pH dependent specific fluorescence appeared in many cells (*cf* Fig 1a) This fluorescence exhibited excitation and emission spectra (375/500 mμ similar to those obtained after acidification from the cells showing fluorescence also after formaldehyde treatment alone (Fig 5)

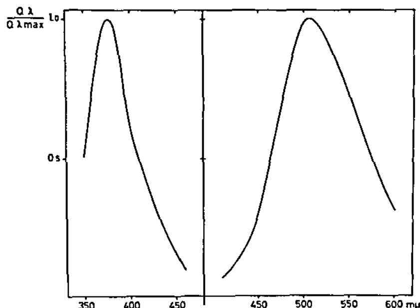


Fig 6 Excitation and emission spectra from a yellow fluorescent cell in the adenohypophysis of the cat after formaldehyde treatment in ozone enriched air at an ozone concentration corresponding to 20 min electric discharge in the vessel (type 2 treatment) No changes in the spectra were seen upon subsequent acidification and alkalinization

Upon alkalinization of the sections (i e treatment with NH_3 vapour) after the conventional formaldehyde treatment the intensity of the cellular fluorescence was significantly decreased and reliable emission spectra—but no excitation spectra—could be obtained only from the yellow fluorescent intermedia cells of the 5 month old pigs The alkalinization caused a shift of their emission maximum to shorter wavelengths giving a broad emission peak with the maximum varying between 455 and 495 mμ After 1—5 min exposure to NH_3 vapour the peak was narrower and had the maximum at about 455 mμ (Fig 4) sometimes with a shoulder at 490—500 mμ The shoulder at 430 mμ was most often recorded and was sometimes prominent (Fig 4)

The microspectrofluorimetric characteristics of the fluorescent cells in the adenohypophysis of the pig and rat were thus very similar to those of the fluorescent cells in the adenohypophysis of the cat (Bjorklund and Falck 1969)

B Formaldehyde ozone treatment (type 2 ozone+formaldehyde gas 1 hr +80° C Also after this treatment the yellow fluorescent cells in the pars intermedia and pars distalis of the rat cat and pig showed very similar microspectrofluorimetric characteristics The excitation/emission maxima were at 375/500 mμ and no variations in the spectra were seen upon acidification or alkalinization (Fig 5 and 6)

Discussion

In a previous study on the cat (Bjorklund and Falck 1969), the presence of a substance capable of forming a fluorophore with formaldehyde was reported to occur in most of the pars intermedia cells and in many cells of the pars distalis and evidence was obtained that this fluorophore did not derive from any of the known biogenic catecholamines (dopamine, noradrenaline adrenaline) or 5 HT The present investigation on the adenohypophysis of the rat cat and pig lends further support to these results *Thus the yellow formaldehyde induced fluorescence in the pars intermedia and pars distalis of all three species showed microspectrofluorimetric characteristics after formaldehyde treatment alone as well as after subsequent acidification or alkalinization that clearly differed from those of the fluorophores of the biogenic catecholamines and 5 HT* Furthermore the marked increase in the fluorescence yield in the pituitary cells upon acidification after the formaldehyde treatment is not seen in the case of these monoamines and the markedly higher fluorescence yield *recorded upon the combined formaldehyde ozone treatment* also excludes them since the fluorescence yields of these amines are significantly lower after this treatment than after treatment with formaldehyde alone (Bjorklund Ehinger and Falck 1968 Bjorklund Falck and Håkanson 1968 Bjorklund and Falck 1969) Moreover only very low concentrations of catecholamines and 5 HT have been found in the pars distalis of the pig pituitary where numerous fluorescent cells occur (Bjorklund Falck and Rosengren 1967)

The microspectrofluorimetric and histochemical characteristics of this monoamine like substance are very similar to those obtained from tryptamine in histochemical models (*i e* the amine enclo ed in protein or sucrose glycine films) and it is possible to distinguish them from those of a number of closely related indole compounds such as tryptophan N methyl tryptamine indole 3 acetic acid 5 hydroxytryptamine (*cf* above) 5 hydroxytryptophan 5 methoxytryptamine 5 methoxytryptophan N N dimethyltryptamine and melatonin (Bjorklund Falck and Håkanson 1968) *Thus there is much histochemical evidence that tryptamine is stored in cells of the* pars intermedia and pars distalis On the other hand preliminary attempts to isolate tryptamine from the pituitary gland of the rat and cat according to Hess and Udenfriend (1959) as modified by Eccleston *et al* (1966) have so far been unsuccessful At present it cannot be excluded that the sensitivity of this method is too low to detect the amount of tryptamine that may be present in the pituitary

How reliable then are the histochemical criteria? It is generally considered that the fluorescence method of Falck and Hillarp possesses a high specificity for certain indole and catechol amines and their immediate precursors (Falck and Owman 1965 Corrodi and Jonsson 1967 Jonsson 1967) The molecular requirements for the Pictet Spengler condensation reaction to take place and for the development of strong visible fluorescence under the histochemical standard conditions are such that only β(3 indolyl)ethylamines and 3 hydroxylated β phenylethylamines will give a useful fluorescence in the histochemical reaction as far as is known at present (Corrodi and Jonsson 1967 Jonsson 1967 This concept finds strong support in a vast number of

studies on different types of tissues and on histochemical model systems In other words, a significant fluorescence that appears when tissues are treated according to the conventional histochemical procedure (*cf e g* Falck and Owman 1965) will most probably be due to substances belonging to those groups The fluorophore in the pituitary cells showed the earlier proposed criteria for a specific monoamine fluorescence (see Dahlstrom and Fuxe 1966) (1) it is induced by exposure to formaldehyde gas, (2) it gives a positive sodium borohydride test according to Corrodi *et al* (1964), (3) it fades upon irradiation with UV light, (4) it is quenched by mounting in water Moreover, the microspectrofluorimetric and histochemical characteristics of the fluorophore were similar to those of the tryptamine fluorophore (in model systems) as judged by the following criteria (*cf* Bjorklund Falck and Håkanson 1968) (1) the fluorescence excitation and emission spectra after conventional formaldehyde treatment, (2) the spectral changes upon acidification and alkalinization after conventional formaldehyde treatment, (3) the changes in the fluorescence yield upon acidification and alkalinization, (4) the increase in fluorescence yield upon combined formaldehyde-ozone treatment, (5) the fluorescence excitation and emission spectra after the formaldehyde-ozone treatment, (6) the spectral changes upon acidification and alkalinization after the formaldehyde-ozone treatment Taken together, these 10 criteria point to the presence of tryptamine or a closely related β(3 indolyl)ethylamine in the pituitary gland

It is well established that tryptamine is formed in the body Tryptamine has been isolated from urine (Davies *et al* 1953 Rodknight 1956 Sjoerdsma *et al* 1959 Donaldson 1962 Gottfries and Magnusson 1963) and this tryptamine was found not to originate from the intestinal bacteria (Sjoerdsma *et al* 1959 Donaldson 1962) The excretion of tryptamine can be markedly increased by administration of a monoamine oxidase inhibitor and as much as 1 to 3 mg of tryptamine has been estimated to be formed and metabolized per day in man under normal conditions (Hess Redfield and Udenfriend 1959) No significant amounts of tryptamine however, have been isolated from normal mammalian tissues (Hess and Udenfriend 1959 Eccleston *et al* 1966) Hess and Udenfriend suggested that this was due to the insufficient sensitivity of the method used in fact after monoamine oxidase inhibition detectable levels of tryptamine were found in the guinea pig liver but not in the brain (Hess Redfield and Udenfriend 1959 Eccleston *et al* 1966) Cell systems normally forming and storing tryptamine have not so far been demonstrated but the possibility that such systems exist in the adenohypophysis should now be considered In this connexion it is of interest that histochemical studies (Matusi and Kobayashi 1965 Dahlstrom and Fuxe 1966) suggest the occurrence of aromatic amino acid decarboxylase and monoamine oxidase in the adenohypophysis which has been confirmed in preliminary biochemical studies on the pig pituitary gland (Bjorklund and Håkanson unpublished observations)

It has been clearly demonstrated that the yield of the tryptamine fluorophore is much higher after the combined formaldehyde-ozone treatment than after conventional formaldehyde treatment presumably because of an increased oxidative c'

hydrogenation of the initial condensation product (Bjorklund Falck and Håkanson 1968) Also acidification after conventional formaldehyde treatment evokes a substantial rise in the fluorescence intensity In fact either treatment produces a final fluorescence intensity from tryptamine which is higher than that obtained from *e g*, dopamine noradrenaline and adrenaline in the Falck—Hillarp method The formaldehyde-ozone reaction and the acidification of tissue sections after formaldehyde treatment have recently been successfully applied on tryptamine containing tissues (submaxillary gland and pancreas incubated in a tryptamine solution) showing that an intense specific fluorescence can be obtained which stands out brilliantly against an essentially dark background (Björklund, Falck and Håkanson 1968) It was also demonstrated that tryptamine concentrations too low to be detected by the Falck—Hillarp technique can easily be detected in these procedures Much argues for the view that the above mentioned pituitary cells store tryptamine and if this be so the applicability of the fluorescence methods according to Bjorklund *et al* (1968) have been demonstrated on storage sites for endogenous tryptamine also at concentrations too low to be detected by the conventional Falck—Hillarp technique only after these treatments could the fluorescent cells in the pars distalis of the adult rat and in the pars intermedia of the 3 month old pig be visualized

The acidification after the conventional formaldehyde treatment also disclosed a second population of fluorescent cells in the pars distalis of the rat and pig the light emitted by these numerous cells being similar to that exhibited by the catecholamine fluorophores Nevertheless this fluorophore cannot derive from any of the hitherto identified biogenic catecholamines (or dopa) the fluorophore yield of adrenaline is low under the reaction conditions used (Falck Haggendal and Owman 1963 Norberg Ritzen and Ungerstedt 1966) and upon acidification of the sections the intensity of the noradrenaline and adrenaline fluorophores will considerably decrease whereas the intensity of the dopamine (and dopa) fluorophores will be only slightly affected (Bjorklund Ehinger and Falck 1968) The substance responsible for this second type of fluorescence has not yet been identified but its presence suggests that monoaminergic mechanisms may operate in more than one cell population in the pars distalis

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The Influence of DOPA on the Static and the Dynamic Fusimotor Activity to the Triceps Surae of the Spinal Cat

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Abstract

Grillner, S *The influence of DOPA on the static and the dynamic fusimotor activity to the triceps surae of the spinal cat* Acta physiol scand 1969 77 490—509

The influence of descending noradrenergic fibres on the stretch reflex and the discharge of γ motoneurones to the triceps surae has been analysed by injecting DOPA i v in the acute spinal unanesthetized cat After DOPA a tonic stretch reflex develops in the soleus muscle as revealed by an increased electromyographical activity on extension of the muscle In parallel static γ motoneurones acquire a resting discharge which can be inferred from the finding that secondary endings have an elevated discharge rate after DOPA The discharge in single γ efferents could be influenced through short latency reflex paths from afferent fibres including group II afferents further the γ-efferents were found to participate in the late and long lasting reflex effects that can be evoked after DOPA On the basis of their resting discharge and reflex effects the γ-efferents recorded could tentatively be identified as static or dynamic Both static and dynamic γ motoneurones to extensors have a higher resting discharge after DOPA Hence in contrast to previous findings on flexor γ motoneurones no reciprocal control on the resting discharge in the two types of efferents is exerted Only γ motoneurones classified as static received monosynaptic activation from fast descending fibres presumably vestibulospinal

An i v injection of DOPA in the spinal unanesthetized cat causes a profound change in the reflex transmission in the spinal cord (Anden *et al* 1966 a Jankowska *et al* 1967 a b Grillner Hongo and Lundberg 1967) The effect of DOPA can with reasonable certainty be ascribed to the release of noradrenaline from descending 'noradrenergic' fibres Anden Jukes and Lundberg 1966 b Jurna and Lundberg 1968) An i v injection of DOPA in the spinal preparation exerts a reciprocal effect on the reflex control and the spontaneous activity in dynamic and static γ motoneurones to flexors in that the reflex effects and the resting discharge is depressed in dynamic but enhanced in static Bergmans and Grillner 1968 a 1969)

The present investigation is concerned with the influence of DOPA on the resting discharge and the reflex control of dynamic and static γ motoneurones to the triceps surae Reflex effects to these motoneurones were investigated previously in detail

by Hunt and Paintal (1958) They found a large variability in reflex effects and concluded that α and γ motoneurones differ considerably in respect to their reflex connections Their study was however, performed before the discovery of dynamic and static γ motoneurones (Jansen and Matthews 1962 a Matthews 1962) In the spinal cat recordings of muscle spindle afferents from extensors suggest that dynamic γ motoneurones, in contrast to static, have a resting discharge and can be reflexly activated by repetitive nerve stimulation (Alnaes Jansen and Rudjord 1965) It will be shown that static γ motoneurones acquire a resting discharge after an injection of DOPA, and that in parallel there occurs a tonic myotatic reflex which is not present in the acute spinal cat but which is well known to occur in the decerebrate preparation (Liddell and Sherrington 1924), when static fusimotor activity is abundant (Jansen and Matthews 1962 a b Jansen 1966)

Methods

24 cats were operated upon under ether anesthesia and subsequently intercollicularly decerebrated or anemically decorticated (Voorhoeve 1960 Anden *et al* 1966 a)

Operation procedure The cats were spinalized at the lower thoracic level and the ipsi and the contralateral ventrolateral funicles were separated and mounted on stimulat on electrodes further a lower lumbar laminectomy (L4—L7) was performed

In the experiments where spindle afferents were studied the ipsilateral (left) hindlimb was denervated (*cf* Bessou and Laporte 1965) except for the nerve to the soleus muscle The plantaris and the late al gastrocnemius muscles were removed The soleus tendon with its bony insertion was separated from the calcaneus The tibia was rigidely fixed at its proximal and distal ends by steel pins drilled into the bone

In the experiments where single γ-efferents were stud ed filaments in the lateral or medial gastrocnemius muscle were d ssected Ips laterally the sural nerve and the nerve to PBSt and

hamstring and the
ft intact The skin
pools which were
was controlled and
and low molecular

dextran (Macrodex R) The blood pres ure was continuously recorded and prevented from fall ng below 90 mm Hg

In the latter type of experiments the cats were artificially respirated and curarized with gallamine triethiod de (Flaxedil®

Isolation of spindle afferents Small filaments were taken from the dorsal roots of L7 which were otherwise left intact The isolation and identification of s ngle spindle afferents were performed conventionally (*cf* Bergmans and Grillner 1968 a) The endings were identified as being of the secondary type by their conduction velocity (< 2 m/sec) (Hunt 1954) The secondary endings recorded from had conduction velocities between 37—50 m/sec

Isolation of γ efferents Single γ efferents were isolated in peripheral nerve filaments to the latera
root
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wave pulses Single stimuli short trains (intervals usually 2 5 or 3 3 msec) or a continuous train of pulses at variable intervals could be delivered to the peripheral nerves Single stimuli or short trains of pulses were never repeated more often than once every 3rd second Activity in the γ efferents or afferent fibres was recorded bipolarly with Ag—AgCl electrodes Afferent volleys were mon tored through a surface electrode at the dorsal root entry zone of the appropriate segment The electromyographical activity in the soleus muscle was recorded between a plat num electrode resting on the surface of the proximal part of the soleus muscle and an indifferent electrode in the crural muscles The record ng electrode was mounted on a weak spring giving an even pressure on the muscle The tension developed in the muscle was measured with a strain gauge in series with the tendon the former was mounted on a manipulator through which the length of the muscle could be changed to any desired value

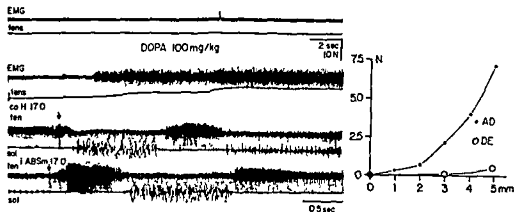


Fig 1 The stretch reflex in the acute spinal cat before and after DOPA Upper traces in the two upper series show the EMG recorded on the surface of the soleus muscle before and after an injection of DOPA and the lower beams the tension in the tendon of the soleus muscle on a slow extension (5 mm) in two steps The lower two series show the activity evoked in a filament (ten) to a flexor muscle (upper traces) and the EMG of the soleus muscle (lower traces) after stimulation of a contra (coH) and an ipsilateral (iABSm) nerve Longlasting alternate flexor and extensor activity is evoked initially extensor from coH and flexor from iABSm The stimulation strength is in this and consecutive figures expressed in multiples of the threshold for the most excitable fibres The graph to the right shows the tension expressed in newton (N) (100 g wt = 0 981 N) in the soleus muscle at different levels of extension (mm) after DOPA (AD) and after de efferentiation (DE) Time and tension calibrations as indicated

Abbreviations ipsilateral i contralateral co ventral root VR, Hamstring nerves H, posterior biceps semitendinosus PBSt anterior biceps semimembranosus ABSm suralis Sur, tenuissimus ten gastrocnemius G soleus S excitatory postsynaptic potential EPSP inhibitory postsynaptic potential IPSP threshold thr millivolt mV newton, N millisecond msec pulses per second pps, Flexor reflex afferents (ie group II and III muscular afferents cutaneous and joint afferents) FRA

Terminology Inhibition will be used for both disfacilitation and postsynaptic inhibition The term flexor reflex afferents (FRA) is used when effects are evoked in common from group II and III muscular afferents cutaneous and joint afferents It does not imply that these afferents always exert the same effect

Results

I *The influence of DOPA on the stretch reflex*

A tonic stretch reflex can be elicited in both the decerebrate and the chronic spinal preparation but not in the acute spinal cat (Liddell and Sherrington 1924 Denny-Brown and Liddell 1927) The two upper series of records in Fig 1 show the EMG recorded on the surface of the soleus muscle and the tension in the tendon of the muscle A slow extension of the muscle evokes no activity prior to an iv injection of DOPA but after DOPA there occurs a gradually increasing electromyographical activity and simultaneously the tension in the muscle is increased The EMG is maintained at a high level as long as the muscle is stretched In Fig 2 the EMG in the soleus muscle is recorded in the lower beam during maintained stretch at different extensions before DOPA (A C) and after DOPA (D—I) In D two

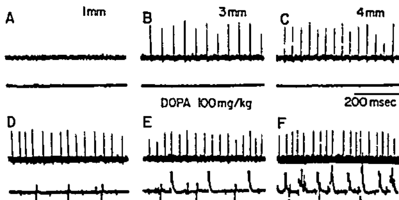


Fig 2 The influence of DOPA on the discharge rate of a secondary ending Upper traces show the activity in a secondary ending and the lower traces show the electromyographical activity recorded on the surface of the soleus muscle The discharge rate and the electromyographical activity is shown at different levels of extension before (A—C) and after an injection of DOPA (D—F)

motor units are active in E and F successively more units are recruited The increase of tension in the tonic stretch reflex of the decerebrate preparation is obtained largely through the recruitment of new motor units and not through a frequency modulation of the active units (Denny Brown 1929 Granit 1958) It is my impression that within a rather wide range this holds true also for the tonic stretch reflex obtained in the present preparation (*cf* Fig 2) In the graph of Fig 1 the tension developed versus extension in the soleus muscle is plotted after an injection of DOPA (AD) and also after the nerve to soleus has been cut (DE) The tension developed after DOPA is dependent on the length of the muscle and is considerably higher than during extension of the passive muscle Hence the muscle must be actively contracting The steeper tension extension relation after DOPA is partly due to differences between the properties of the active and the passive muscle fibres (*cf* Granit 1958 Matthews 1959 a) but also to the increased motoneuronal activity (*cf* EMG in Fig 1 2)

A tonic myotatic reflex could be demonstrated in six out of seven preparations In all but one (Fig 1) the presence of a tonic stretch reflex was investigated simultaneously with the recording of spindle afferents from the muscle For this purpose parts of the dorsal roots were cut This might explain the lack of effect in one cat The stretch reflex obtained varied sometimes from one trial to the other For these reasons no attempt was made to evaluate systematically the static or dynamic gain of this myotatic reflex Furthermore the gain of the stretch reflex should presumably be estimated by stretching the muscle together with its synergists since the amount of heteronymous Ia excitation from synergists at the same joint is con able (Eccles Eccles and Lundberg 1957 c)

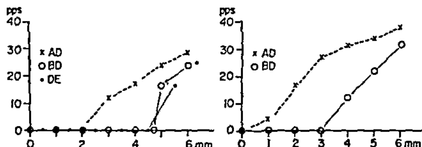


Fig 3 The frequency—extension relation of two secondary endings before and after an inje tion of DOPA (100 mg/kg) The discharge frequency (ordinate) of the secondary endings a plotted versus the extension of the soleus muscle (abscissa) before (BD) and after (AI DOPA and after an intravenous injection of Flaxedil® of 8 0 mg/kg blocking the intrafus neuromuscular transmission (DE)

II *The influence of DOPA on the discharge of secondary endings*

The tonic myotatic reflex occurring in the soleus muscle after DOPA might b related to an enhanced activity in the γ-loop and it is of particular interest to kno if any changes occur in the static fusimotor activity, since only dynamic γ moto neurones appear to have a resting activity in the spinal cat (Alnaes *et al* 1965 Since secondary endings are influenced only from static γ-motoneurones (Appelberg Bessou and Laporte 1966, *cf* Brown Engberg and Matthews 1967) changes of th discharge in secondary endings provide information indirectly on the activity o static γ motoneurones

Fig 2 shows a secondary ending at three different levels of extension before DOPA (A—C) and after (D—F) In A there is no resting activity but at the same muscl length in D the ending has acquired a discharge of 23 pps The discharge rate enhanced also at the other levels of extension (B, E, C, F)

The frequency extension relation of two secondary endings is plotted in Fig before (BD) and after DOPA (AD) The endings start firing at lower extension (2 8 and 3 mm respectively) after as compared to before DOPA At all extension the firing rate is higher after DOPA but the difference is smaller at higher exten sions which presumably is due to the development of an increased extrafusal ac tivity In the diagram to the left also the discharge rate after an injection of Flaxed (DE) at a dosage blocking the intrafusal as well as the extrafusal transmission (Hun 1952) is shown The discharge rate is not appreciably different under de-efferente (DE) conditions and before DOPA (BD) *i e* in the normal spinal cat

Five out of six secondary endings showed an increased discharge rate after DOPA Since this increase could be observed also when there was no sign of electromyo graphical activity in the muscle it is unlikely to be caused by mechanical interac tion but is rather due to an enhanced fusimotor activity The α innervation o muscle spindles Bessou Emonet Denand and Laporte 1965) seems mainly to in fluence the dynamic sensitivity of primary endings The effect on secondary endings, however is not known Since acceleration of secondary endings has also been ob-

tained without apparent EMG activity the most likely explanation is that there is an increase of the resting discharge in static γ-motoneurones since the secondary endings are not influenced from dynamic γ-motoneurones (Appelberg *et al* 1966 *cf* Brown *et al* 1967) Furthermore, α innervation of muscle spindles within the soleus muscle seems to be comparatively rare (Brown Crowe and Matthews 1965) The similarity between the discharge rate before DOPA and under Flaxedil indicates that there is no or a low level of activity in static γ-motoneurones in the spinal cat (*cf* Alnaes *et al* 1965)

III *Recording from single γ efferents*

a *Resting activity*

There is conflicting evidence concerning the existence of a resting activity in fusimotor neurones supplying extensor muscles in the spinal cat Thus the results of Hunt and Paintal (1958) and Alnaes *et al* (1965) indicate that there is a resting activity in part of this group of γ-efferents but this has been denied by Diete Spiff, Dodsworth and Pascoe (1962) and Voorhoeve and van Kanten (1962)

In the present series of low spinal unanesthetized preparations a resting activity was usually encountered when recording from filaments before the isolation of single units A resting activity was observed in 4 of 15 recorded single units with a frequency of 40, 22 20 and 8 pps

Since the level of resting activity can be influenced from a wide receptive field and by rather unspecific stimuli (cf below) the possibility that the resting activity could be due to the rather extensive operative wounds had to be considered To test this one cat was spinalized and only a minimal incision was made to expose a filament of the lateral gastrocnemius muscle The incision in the skin was infiltrated with xylocain Nevertheless a resting activity was encountered in several units of this filament and also in other filaments which were later exposed Hence in confirmation of Hunt and Paintal (1958) these results indicate that there is a resting activity in part of the γ efferents to gastrocnemius in the spinal preparation

In Fig 4 two units (A—C D—F) one with no resting activity and another with a resting discharge rate of 20 pps are shown (B E) After an intravenous injection of DOPA both units are accelerated to 85 and 45 pps respectively In the diagram on the right hand the resting discharge rates of all γ efferents investigated are presented before and after an injection of DOPA All units except one which was not influenced were accelerated after the injection of DOPA Hence in contrast to flexor γ motoneurones (Bergmans and Grillner 1968 a 1969) there is no indication of two types of γ efferents in which the spontaneous activity is influenced in an opposite direction following the injection of DOPA

The relative regularity of a resting discharge is conveniently expressed as the coefficient of variation (CV) i e standard deviation over the mean for the duration of successive interspike intervals Stationarity of the discharge was checked for by plotting the duration of successive intervals (n>50) The CV for seven units ranged between 0 14—0 24 The CV did not change after DOPA in three of the units tha

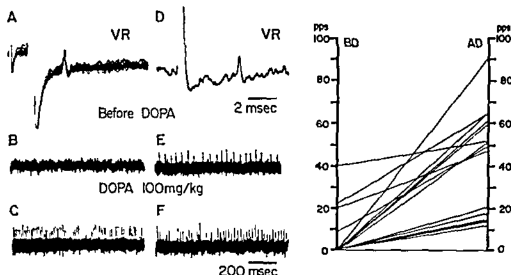


Fig 4 Resting activity in γ efferents before and after DOPA A—F show two γ efferents identified by stimulation of the ventral roots (A, D), one with no resting discharge (B) and another with a discharge of 20 pulses per second before DOPA (E) Both efferents are accelerated after the injection of DOPA (C, F) The graph to the right shows resting discharge rate before and after the injection of DOPA for the 15 γ efferents recorded

had a resting discharge before DOPA, although the resting discharge increased The interval histograms were either symmetric or had a slight degree of positive skew At the end of the experiments the dorsal roots of L5 to S2 were usually transected bilaterally The discharge frequency was decreased but not entirely abolished, the CV and the degree of positive skew, however, increased

b *Reflex effects evoked with short central delay*

Previous investigations of the reflex connexions to γ-motoneurones, particularly those distributed to extensors have not yielded any clear-cut pattern (Hunt and Paintal 1958) A fraction of the γ-motoneurones to the medial gastrocnemius muscle have no background discharge (Hunt and Paintal 1958) in the high spinal animal In this group several neurones could not be excited by electrical stimulation of any peripheral nerve and the interesting possibility that there are no short latency pathway to these neurones has been considered Whether a cell with no resting discharge is in fact excited and subthreshold for evoking a spike, inhibited or provided with no reflex connexions can only be tested with intracellular recording or after the inducement of a resting discharge

Cells with a resting discharge (4/15) could all be influenced after a short segmental latency through excitatory or inhibitory (or disfacilitatory) reflex paths Of the remainder a reflex discharge could be evoked in five neurones but in six no reflex effects were obtained although stimulation strengths with single and repetitive shocks (3—5) up to 50 times threshold for the most excitable fibres were always

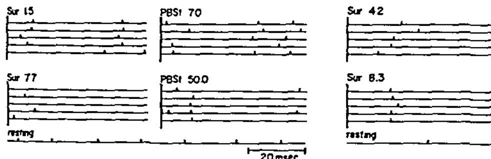


Fig 5 The reflex activation of two γ-efferents after DOPA that could not be reflexly activated and neither had a resting discharge before DOPA. The discharge of a γ-efferent is schematically represented, each horizontal line indicates one sweep (time) and each vertical bar the occurrence of one spike. The stimulation is set up in the beginning of each sweep (large vertical bar). The two left columns show one unit spontaneously discharging in which the resting discharge is inhibited from the sural nerve and the nerve to PBSt. The column to the right shows a unit with a lower activity which is activated on stimulation of the sural nerve

tested. After DOPA short-latency reflex effects could be evoked in all γ motoneurones, which presumably is related to the occurrence of a resting discharge (Fig 4).

Fig 5 shows the reflex effects to two γ motoneurones that could not be influenced before DOPA. Each horizontal line represents one sweep (time) with the stimulation set up in the beginning. Each vertical bar represents one action potential. In the period between 10—35 msec after the stimulation of the sural nerve no spike occurs although the mean interval is only 15 msec and at higher strength the silent period has a longer duration. Inhibitory effects are evoked also by stimulation of a muscle nerve (PBSt). At higher strength the spikes preceding the discharge interval had a very stable latency suggesting that the inhibitory period was preceded by an excitation. The series to the right are from another cell that was activated at a rather variable latency at a stimulation strength of 4.2 times threshold from the ipsilateral sural nerve but at a more stable latency at 8.3 times threshold.

γ motoneurones can be influenced from cutaneous and muscular afferents and also from joint afferents (Hunt and Paintal 1958, Grillner, Hongo and Lund 1969). That group II muscular afferents contribute to these effects was stated by Voorhoeve and van Kanten (1962) and this has been confirmed for flexor γ motoneurones by Bergmans and Grillner (1969). Fig 6 shows a G—S γ-motoneurone that receives excitation from ipsilateral nerves (B—D) but no evident effects from the contralateral side. The graph to the right shows the firing index

$$\left(\text{i.e.}\ \frac{\text{no. of reflex responses}}{\text{no. of trials}} \times 100\right)$$

plotted versus stimulation strengths of the nerve to ABSm expressed in multiples of the threshold for the most excitable fibres, stimulated with single and ... ve

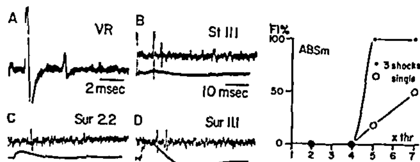


Fig 6 The reflex effects evoked from group II and other afferents Upper traces show the records from one γ efferent (before DOPA) in a filament to the lateral gastrocnemius (cf A) The lower traces show the activity recorded at L7 dorsal root entry zone The efferent is discharged from the sural (C D) and the semitendinosus nerve (B) The graph to the right shows the firing index (cf text) plotted versus stimulation strength expressed in multiples of the threshold for the most excitable fibres of the nerve to ABSm The firing index is shown with single stimulation and after three shocks and is based on 10 observations except at single stimulation at 5 × thr when only six observations were made

stimuli (3 shocks) The cell is not activated at 4 times threshold, but in 100 % of the cases at 5 times threshold with 3 shocks, but only at 15 % with single stimuli The firing index is increased to 50 % at 7 times threshold showing that afferent fibres activated in this range contribute to excitation Since group II afferents are activated at a range between 2 5—8 times threshold (Eccles and Lundberg 1959) these results demonstrate that γ-motoneurones can be influenced from afferents conducting in the group II range However it should be noted that these afferents might not be entirely composed of spindle afferents with secondary endings (cf Barker 1967) In most γ-efferents excitatory or inhibitory reflex effects could be revealed either before or after DOPA at group II strength

Coactivation of α and γ efferents is shown in Fig 6 B from the nerve to PBSt At high stimulation strengths both were always activated thus demonstrating an α γ-linkage in reflex effects although the pattern is not that of the flexor reflex Even at higher strengths only one spike was evoked in the γ efferent from the PBSt Although stimulation of the sural nerve elicited two spikes in the γ efferent the α motoneurone was not discharged Hence the synaptic effects from the two nerves to these α and γ motoneurones were not distributed exactly in parallel In all filaments recorded from at least one α efferent was present but only in three cases were these efferents discharged from ipsilateral muscular or cutaneous nerves thus indicating an α γ linkage In the other filaments when excitatory effects were revealed in the γ motoneurone the α motoneurones were not discharged which might be due to differences in reflex organization or merely reflect a higher threshold for activation

In the nerves from the thigh muscles Ib afferents have a higher threshold for activation than Ia afferents (Bradley and Eccles 1953 Eccles Eccles and Lundberg 1957 a) and by careful grading of the stimulus strength the activation of Ia fibres can be separated from Ib One γ-efferent receiving excitatory action mainly from ipsilateral cutaneous and muscular nerves ~ activated from the nerve to semitendinosus *The firing index was successively increased*

It was more difficult to elicit reflex effects from the contralateral hamstring and sural nerves than from ipsilateral nerves. The γ-efferents tested were rarely activated at a stable latency but more often the stimuli were followed by excitatory effects revealing themselves as a slight shortening of the interspike intervals of a few successive spikes, probably being due to synaptic effects with a gradual onset and cessation. Inhibitory effects could be evoked in a few cells. It should be noted that the crossed extensor reflex to the gastrocnemius is usually very small (Eccles and Granit 1929) and that excitatory crossed reflex effects to extensor motoneurones were present only in 50 % of the acute spinal preparations tested by Holmqvist (1961), in the remainder no, or inhibitory effects were evoked. These experiments were, however, performed with monosynaptic test reflexes that presumably give a preference for testing slow motor units, since their motoneurones receive larger EPSPs (Eccles, Eccles and Lundberg 1957 c). This might be of importance since differential effects from ipsilateral cutaneous nerves can be exerted on slow and fast motor units (cf. Discussion below).

In the majority of γ-motoneurones a larger reflex response could be elicited after DOPA. In two neurones, however, spontaneously active (20–40 pps) before DOPA the reflex effects were less pronounced after DOPA. Since mixed effects (cf. Fig. 5) were often encountered, a depression of the reflex response might not reflect a decreased efficiency in that particular pathway but the facilitation of a pathway with opposite effect. Finally in some instances the reflex effects elicited at low strength seemed to be depressed after DOPA but the reflex discharge at higher strengths could be enhanced.

c. *Repetitive nerve stimulation*

Alnaes, Jansen and Rudjord (1965) activated dynamic but not static γ motoneurones with repetitive nerve stimulation at 40 pps in the spinal cat. Repetitive stimuli at different frequencies have differential effects on static and dynamic γ motoneurones to flexors (Bergmans and Grillner 1969). Extensor γ motoneurones that could not be activated with single shocks or a short train of pulses could neither be activated by longlasting repetitive stimulation at different frequencies, only occasionally a few spikes were elicited.

In Fig. 7 the discharge frequency is plotted versus stimulation frequency. The left graph (A) shows the response of a spontaneously discharging unit to stimulation of the contralateral sural nerve. There is a positive correlation between discharge frequency and stimulation rate. On the other hand, a unit reflexly activable with single stimuli to coSur but with no resting discharge is shown in B, where the discharge frequency is enhanced to 40 pps at a stimulation frequency of 10, but is decreased to

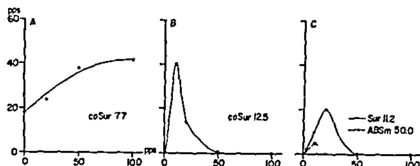


Fig 7 The effect of repetitive nerve stimulation on the discharge of single γ efferents before the injection of DOPA The discharge frequency (ordinate) is plotted versus the frequency of stimulation The nerves stimulated and the stimulation strength is indicated in the different graphs Only the unit in A had a resting activity

15 pps at a frequency of 20 and is silent at 50 pps Corresponding effects are shown in C for a unit reflexly excited from ipsilateral nerves In γ motoneurones with a resting activity before DOPA (3 tested) there was a positive correlation between discharge and stimulation frequencies from nerves giving excitatory effects , motoneurones without resting discharge were not or were very poorly activated at the stimulation frequency employed by Alnaes *et al* (1965) (Fig 7 B C)

d *Adequate activation*

Different regions of the hairy skin were activated by gentle stroking and stronger stimuli applied by pressing the skin with underlying tissues between the fingers 2/3 of the γ motoneurones were accelerated from the contralateral paw and decelerated from the ipsilateral thus conforming to the flexor reflex pattern In the remainder acceleration was obtained from the ipsilateral paw Since the pattern of activation was generally not changed after DOPA the neurones that had no resting discharge and could not be influenced before DOPA were tested against the background discharge obtained after DOPA If the receptive fields were studied more in detail more subtle patterns could sometimes be revealed For instance acceleration could be obtained from the pads and sometimes from one particular toe pad but inhibition from the hairy region proximal to the main pad

Both cells activated from the contra and the ipsilateral paw were often activated from the skin overlying the ipsilateral iliac crest A few γ motoneurones tested more extensively could be influenced by manipulation of the muscles from the level of the spinalisation and further caudally also from the periosteum of the vertebrae the iliac crests and sacrum Even pressure applied to the last few millimetres of the tail influenced the discharge rate as well as manipulation of the rectal thermometer and an increased pressure in the bladder in confirmation of Abdullah and Eldred (1959) and Evans (1963

It is difficult to find any particular meaningful pattern in this modification of discharge rate obtained from such a large receptive field (*cf* discussion)

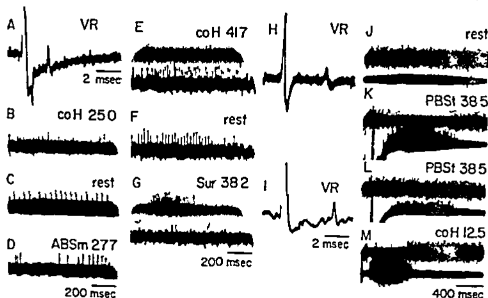


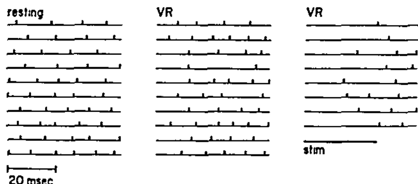
Fig 8 Longlasting reflex effects after an injection of DOPA (100 mg/kg) The discharge of three γ-efferents to the lateral gastrocnemius recorded in different experiments (A—D E—G (lower traces) I J—M (upper traces) H) and the simultaneous discharge in a flexor filament (ten E G St J—M) is shown during resting conditions rest and after stimulation of contra (co) and ipsilateral nerves Before DOPA unit A had no resting discharge in contrast to H and I

e *Longlasting reflex effects evoked after DOPA*

After DOPA a reciprocally organized longlasting discharge can be evoked in flexor and extensor motoneurones usually organized in the flexor crossed extensor reflex pattern described by Sherrington (1910) (Jankowska *et al* 1967 a) and under certain conditions alternate activation similar to spinal stepping can be initiated (Jankowska *et al* 1967 a *cf* also Fig 1)

In Fig 8 the reflex effects evoked in three gastrocnemius γ motoneurones are shown The efferent in the left column is accelerated from the contralateral hamstring (coH) nerve as compared to the resting state (C) but on stimulation of the ipsilateral nerve to ABSm (iABSm) the resting discharge is abolished In the second column the simultaneous effects evoked in a flexor filament (ten) and a single unit in an extensor filament are recorded A reflex discharge is evoked from coH and inhibition from iSur coinciding with a late reflex discharge in the flexor filament In the right column the reflex effects evoked in another pair of flexor (St) and extensor filaments is shown from another preparation Records K L are evoked with the same stimulus strength in consecutive records from iPBSt The resting activity in the extensor filament is totally abolished during the large flexor discharge in K but decreased only to a limited degree during the smaller flexor discharge in L. This suggests that the reciprocal excitatory and inhibitory effects are linked to each other

Although the flexor crossed extensor reflexes are usually encountered



patterns are not altogether exceptional (Jankowska *et al* 1967 a) In M a flexor discharge is evoked from coH, which, however, coincides with an abolition of the discharge in the extensor γ-efferent

The prominent pattern has been that of inhibition of the resting discharge from ipsilateral nerves and usually acceleration from contralateral nerves in γ motoneurones with and without a resting activity before DOPA However, other reflex effects were also observed (*cf* Fig 8 M) A relatively short lasting reflex discharge in the extensor γ efferents preceding the late flexor discharge was quite frequently observed (*cf* Fig 12 F G in Jankowska *et al* 1967 a)

From intracellular studies it has been postulated that the reciprocal effects to flexor and extensor motoneurones are exerted at an interneuronal level This inhibition of the resting discharge in γ-efferents was not observed by Jankowska *et al* (1967 a) but must either be exerted in the motoneurones or interfere with the interneurones mediating the tonic excitation responsible for the resting activity

f Recurrent effects

Recurrent inhibitory effects on γ motoneurones have recently been demonstrated by Ellaway (1968) and Brown Lawrence and Matthews (1968)

Recurrent effects were investigated in 9 cells always after injection of DOPA which provided a background discharge The recurrent inhibitory effects to α motoneurones are more pronounced after DOPA (Andén *et al* 1966 a) Fig 9 is a schematic representation of the discharge in a γ motoneurone, each vertical bar representing the occurrence of one spike (*cf* Fig 5) The series to the left shows the resting discharge in consecutive records and the middle series the discharge pattern after a single shock applied to the intact ventral root at a stimulation strength just below threshold for activation of the -efferent Each sweep starts 4 5 msec after the stimulus was applied corresponding to the conduction time from the ventral root to the filament thus excluding action potentials elicited in the soma before the shock was applied A reduced number of spikes occur during the first 15 msec as compared

to resting conditions In the series to the right a repetitive stimulation at (400 pps) is applied and the discharge rate is considerably lowered Recurrent inhibition was found in four of nine cells and was tested for either with the above method or by stimulation of that part of the ventral root that had been cut during the isolation of the γ-efferent (*cf* Methods) *The inability to demonstrate recurrent inhibition* does not exclude the presence of recurrent connections not revealed by the methods employed

IV *Descending monosynaptic activation of γ motoneurones*

With intracellular techniques it has been demonstrated that monosynaptic EPSPs can be evoked in G S γ motoneurones from the region of Deiters nucleus by fast conducting fibres presumably vestibulospinal (Grillner *et al* 1969)

In the present experiments all cells that had no resting discharge could be activated from the ipsilateral ventrolateral funicle with a latency of 1 2—2 7 msec when the conduction time from the stimulation electrodes to the segment and from the ventral root to the filament had been subtracted This latency includes not only the delay between the arrival of activity in the descending fibres to the onset of EPSP, which in the intracellular material of Grillner *et al* (1969) varied between 0 4—0 9 msec but also the time for impulse initiation and the conduction time to *the point of stimulation of the ventral root The minimal time for impulse initia*tion has been estimated to be 0 3 msec and the conduction time 0 4 msec (Bergmans and Grillner 1968 b) Thus a latency of 1 6 msec would correspond to a segmental delay of 0 9 msec Six γ motoneurones activated with a latency between 1 2 to 1 6 msec were assumed to be monosynaptically activated Four motoneurones could not be activated although tested with both single and repetitive stimuli against a high *level of background activity These 4 neurones had a resting activity before DOPA* of 40 22 20 and 8 pps

Discussion

Tentative identification of γ efferents in terms of dynamic and static

The classification of efferents to flexors as static or dynamic was aided by the reciprocal effect of an intravenous injection of DOPA on the resting discharge and by the differential effects of repetitive nerve stimulation (Bergmans and Grillner 1969)

A more complex situation prevails with the extensor motoneurones in which acceleration of the resting discharge was found in virtually all of them after DOPA The secondary endings were discharging at the same level before DOPA and in the de efferented state (Fig 3 A) indicating that there was no or very little static fusimotor bias (*cf* page 5 Appelberg *et al* 1966) after DOPA the discharge rate was at a higher level (Fig 2 3) *showing that static γ efferents had acquired a resting* discharge With indirect methods Alnaes *et al* (1965) showed that in their spinal anesthetized preparation spontaneous activity and reflex activation by repetitive nerve stimulation occurred only in dynamic motoneurones In the present study γ efferents with a resting discharge before DOPA were also further accelerated by repetitive nerve stimulation (Fig 7 A) The remainder with no resting discharge

were not activated significantly by stimulation with the parameters employed by Alnaes *et al* (1965) (Fig 7 B C) Thus in analogy with the results of Alnaes *et al* (1965) it is tentatively suggested that the four neurones which had a resting discharge before DOPA were of the dynamic type and that the other group of neurones were of the static type This interpretation should be further tested by experiments assessing the dynamic sensitivity of primary endings before and after DOPA and after de efferentation

The spontaneous discharge in γ motoneurones

The present results suggest that the resting activity in γ-motoneurones to gastrocnemius, of both the static and the dynamic type is enhanced after DOPA (Fig 4) In contrast DOPA exerts a reciprocal influence on the discharge in γ efferents to flexors in that the activity of dynamic γ motoneurones is depressed but that of static γ motoneurones enhanced (Bergmans and Grillner 1968 a 1969) This difference between flexor and extensor muscles is very interesting and might be related to the different functions of these muscles During muscular shortening (contraction) the activity in primary endings is mainly influenced by the static γ bias but during lengthening of the dynamic γ bias (Lennerstrand 1968 a b) It must be of importance for the antigravity muscles that an increased load resulting in muscle lengthening can be rapidly counteracted the dynamic fusimotor activity creates optimal conditions for a motor regulation of this type

The resting activity in γ efferents of the spinal cat is maintained largely through the activity in the dorsal roots (Hunt 1951) although some activity might persist after transection of the roots (Voorhoeve 1960 Lindsley and Eldred 1960) In the present series the resting discharge decreased but was rarely totally abolished after transection of the dorsal roots L5—S2 Since the receptive field is very wide the de afferentation may not have been complete Hence afferent inflow from this region may have contributed to the resting discharge

It is difficult to see the significance of particular reflexes from the most caudal part of the tail the bladder the muscles at lower thoracic level and from the periosteum of the vertebrae to gastrocnemius γ motoneurones An increasing amount of evidence indicates that the pyramidal (Lundberg and Voorhoeve 1962) and the rubrospinal tract (Hongo Jankowska and Lundberg 1969) as well as other descending systems exert their motor effects through facilitation and inhibition of various segmental reflex arcs These wide receptive fields might simply provide tonic excitation of interneurones having excitatory or inhibitory effects on γ motoneurones thus providing a background activity through which the supraspinal structures can change the discharge of motoneurones through facilitation or inhibition of reflex arcs

It should be emphasized that even if the above explanation is correct it does not exclude the existence of specialized and very delicate spinal reflexes being separated from the above pattern

The mechanism controlling the resting discharge of the γ motoneurones is how

ever not evident Since the spontaneous activity is maintained largel through the afferent inflow from the dorsal roots it can only be concluded that the transmi sio in the reflex arcs must be changed in a manner causing an increased excitab o extensor γ-motoneurones

The reflex effects with short latency

a and , motoneurones of the dynamic and the static type to flexors are discharged in parallel from iFRA in the spinal cat Based on certain differences in reflex re sponse under various conditions it was suggested that these reflex effects were mediated by different reflex paths (Bergmans and Grillner 1969)

Interneuronal transmission from the FRA to extensor a motoneurones is depressed after DOPA (Anden *et al* 1966 a) In the γ motoneurones tentatively classified as static reflex effects could always be revealed after DOPA Since the presence of a resting discharge reflects an increased excitability level no conclusions can be drawn regarding the possible influences on interneuronal reflex transmission The mixed excitatory and inhibitory reflex effects further complicates the interpretation of the effects on reflex transmission to γ motoneurones It is however of some interest that the effects of repetitive nerve stimulation differ in the neurones tentatively identified as static and dynamic

a and , motoneurones to G S are influenced in parallel from certain ipsilateral skin regions (Eldred and Hagbarth 1954) On electrical stimulation Hunt and Pain tal (1958) often found excitatory effects to single efferents to gastrocnemius from both muscular and cutaneous afferents The present study has confirmed these results Their conclusion that γ motoneurones differ in their reflex connexion from that of a motoneurones assumed to be part of the flexor-crossed extensor reflex (Sherring ton 1910) deserves however further discussion

Reflexes of ipsilateral extension other than the stretch reflex were summarized by Creed *et al* (1932) Denny Brown in this monograph (p 73) reports reflex excita t on from the ipsilateral peroneal nerve to the pale gastrocnemii but inhibitory effects from the same stimulus to the red soleus This pattern has recently been confirmed in the spinal cat by Bruggencate Burke and Jankowska (unpublished) showing with intracellular technique that slow motor units regardless of whether in soleus or in the gastrocnemii receive predominantly inhibition from ipsilateral cutaneous nerves but the fast motor units on the other hand receive mainly excitation In the spinal anaesthetized cat extensor motoneurones are usually inhibited from ipsilateral group II and III muscular afferents (Eccles and Lundberg 1959) but in the unanaes thetized preparation the pattern is more variable and excitatory effects are often encountered (Lundberg personal communicat on) Also the reflex effects from contralateral FRA to a motoneurones is rather variable (*cf* page 10 Holmqvist 1961) These findings might be related to the demonstration that there are indeed both excitatory and inhibitory reflex pathways from FRA to both extensor and flexor a motoneurones which are separately controlled from the brain stem (Holm qvist 1961 Holmqvist and Lundberg 1961)

In the present material α and γ motoneurones were sometimes discharged in parallel but in the other cases it cannot be decided whether parallel effects were actually exerted on the α motoneurones or not The presence of slow and fast α motoneurones with different reflex effects within the gastrocnemii (*cf* above) certainly complicates the issue It might be relevant to note that the filament to lateral gastrocnemius generally employed (11/15) supplies the most posterior (superficial) part of the muscle

Contrary to previous findings (Granit Pascoe and Steg 1957), it has recently been shown that some γ motoneurones may be inhibited by impulses in recurrent collaterals of motor axons (Ellaway 1968 Brown *et al* 1968) The recurrent inhibitory effects found in the present series (4/9) were exerted in γ motoneurones tentatively identified as being of the static and the dynamic type Furthermore group I afferents have recently been shown to evoke IPSPs in γ motoneurones It could not be shown whether these effects originated from Ia or Ib afferents but the distribution was similar to the Ib pattern of α motoneurones (Grillner *et al* 1969)

It can by no means be concluded that segmental reflexes are always exerted in parallel to α and γ motoneurones of the same muscle but the only definite difference is the lack of monosynaptic EPSPs from homonymous Ia afferents in γ motoneurones (Hunt and Paintal 1958 Eccles *et al* 1960)

The reflex discharge evoked with long latency

It has been proposed that the neuronal systems responsible for the late reflex discharge in α and γ efferents to flexors and extensors are engaged in stepping and it has been shown that alternate activation of flexors and extensors can be evoked under these conditions (Jankowska *et al* 1967 a *cf* Fig 1) In the late reflex to flexor muscles both α and γ motoneurones of the static and the dynamic type participate (Bergmans and Grillner 1969) On basis of the above tentative identification of dynamic and static γ motoneurones it is suggested that all three types of motoneurones participate also in the late reflex discharge in extensors (Fig 9)

The decrease of the resting discharge (Fig 9 D G K) in these two types of γ motoneurones during the late reflex in flexors is interesting since the removal of γ bias on the spindles in the extensor muscles will result in a decreased activity in spindle afferents from these muscles during the lengthening imposed on the extensors during the reflex contraction of the flexors and hence reduce the reciprocal Ia inhibition from extensors to flexors These results do not allow any conclusion of whether this inhibition of the resting discharge is caused by postsynaptic inhibition of the γ motoneurones or whether it is due to a depression of the interneuronal system responsible for the resting activity

The myotatic reflex

No systematic change in excitability of α motoneurones was found after the injection of DOPA in the spinal preparation employed by Andén *et al* (1966 a) in which most peripheral nerves to the hindlimb was cut and thus the γ loop dis

connected These findings correlated with the presence of a myotatic reflex under resting conditions after DOPA show that the excitability level of the homonymous α motoneurones in the present preparation is largely dependent on the muscle length, presumably the activity in the 'γ loop' is of paramount importance (*cf* Matthews 1959 b)

In the decerebrate preparation static fusimotor activity is abundant (Jansen and Matthews 1962 a b Jansen and Rudjord 1965 Jansen 1966), and it has been postulated that the static fusimotor system is entirely dependent on descending facilitatory effects from the brain stem (Alnaes *et al* 1965) After the presumed selective activation of descending noradrenergic fibres by the injection of DOPA the tonic myotatic reflex occurs in parallel with the static fusimotor activity, which elevates the discharge level of the Ia afferents and very likely also increases the static sensitivity to muscle stretch (Jansen and Matthews 1962 a Brown Lawrence and Matthews 1969) Hence the autogenetic excitation from the primary endings would be increased after DOPA It is thus very likely that the tonic myotatic reflex is directly related to the activity in static γ efferents which however, also influences the discharge of secondary endings These might also be of importance for the magnitude of the strech reflex (Laporte and Bessou 1959 Matthews 1969)

Descending monosynaptic activation

An analysis of the descending effect to flexor motoneurones suggests a monosynaptic excitation of static but not of dynamic motoneurones (Bergmans and Grillner 1968 b, *cf* Grillner *et al* 1969) A similar differential control of extensor γ motoneurones is suggested by the finding that while 6 units, tentatively classified as static, received descending activation with a segmental latency suggesting a monosynaptic linkage no corresponding effect was found in any of the four classified as dynamic The absence of a discharge does not exclude monosynaptic EPSPs subthreshold to evoke a spike The test was however performed against a high level of background discharge and with repetetive shocks

These results thus support the suggestion by Grillner *et al* (1969) that descending fibres presumably belonging to the vestibulospinal tract activate mainly static γ motoneurones

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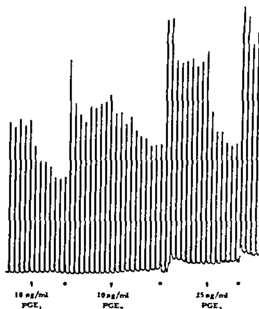


Fig 1 Vas deferens, guinea pig 20 ml bath 37° Transmural stimulation 20/sec, duration 0 8 msec, 5 sec per 1/2 minute Additions to the bath of PGE_1 and PGE_2 as indicated final concentrations Washing at dots

Inhibition of the effect of transmural nerve stimulation was still observed at a concentration of 2 ng/ml A slight increase in the basal tone of the preparation was often seen even in this low concentration

Larger concentrations of PGE_1 of the order of 100 ng/ml (3×10^{-7} M), caused a slight contraction of the vas deferens and increased the response to electrical stimulation

Atropine 10^{-7} g/ml was found to abolish the inhibitory action of PGE_1, without preventing the response to nerve stimulation or the direct effect of NA or PGE_1 on the muscle

The direct contractile response to NA 0 25 µg/ml ($1\ 5\times10^{-6}$ M) in the absence of transmural nerve stimulation was slightly enhanced by PGE_1 2 ng/ml (6×10^{-9} M) confirming the observations of Graham and Al Katib (1967)

Comment

The present observations of an inhibitory effect of low concentrations of PGE_1 and PGE_2 on the contractile response of the isolated vas deferens from the guinea pig to transmural (postganglionic) stimulation are in harmony with the previously reported findings of Brundin (1968) Hedqvist and Brundin (1969) and of Hedqvist (1969 a b) on other adrenergically innervated preparations The increase in tone of the preparation observed even with low concentrations of PGE_1 and their enhancing effect on the contractile response to added NA seem to exclude an inhibitory action directly on the effector cells

Sjöstrand and Swedin (1968) have reported similar findings on some occasion with hypogastric nerve stimulation of the vas deferens Using higher concentration of PGE_1 (100—200 ng/ml) Mantegazza and Naimzada (1965) observed an enhancement of the mechanical response of the vas deferens of the guinea pig to hypogastric nerve stimulation We have confirmed this effect with transmural stimulation

The annulment after atropine 10^{-7} g/ml of the inhibitory action of PGE_1 on the contractile response to transmural nerve stimulation may indicate involvement of a cholinergic mechanism (*cf* Burn and Rand 1959, Burn 1966)

The results reported in this communication suggest that PGE_1 and PGE_2 in oligodynamic concentrations inhibit the transmitter release from adrenergic nerve terminals There is no evidence to show that prostaglandins act as conduction blockers (*cf* Holmes, Horton and Main 1963)

The finding that PGE_2 is released by sympathetic nerve stimulation to the spleen (Davies, Horton and Withrington 1967) indirectly supports the concept that PGE acts as a modulator of adrenergic nerve transmission, acting by a negative feedback mechanism (Hedqvist 1969 a)

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